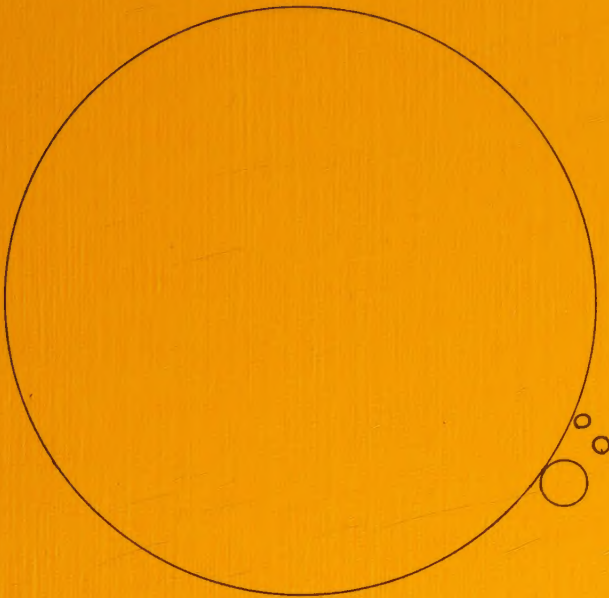




PARTICLE INDUCED X-RAY EMISSION -
ANALYSIS OF BIOLOGICAL SAMPLES

Jan Pallon

DEPARTMENT OF NUCLEAR PHYSICS, LUND INSTITUTE OF TECHNOLOGY, 1986

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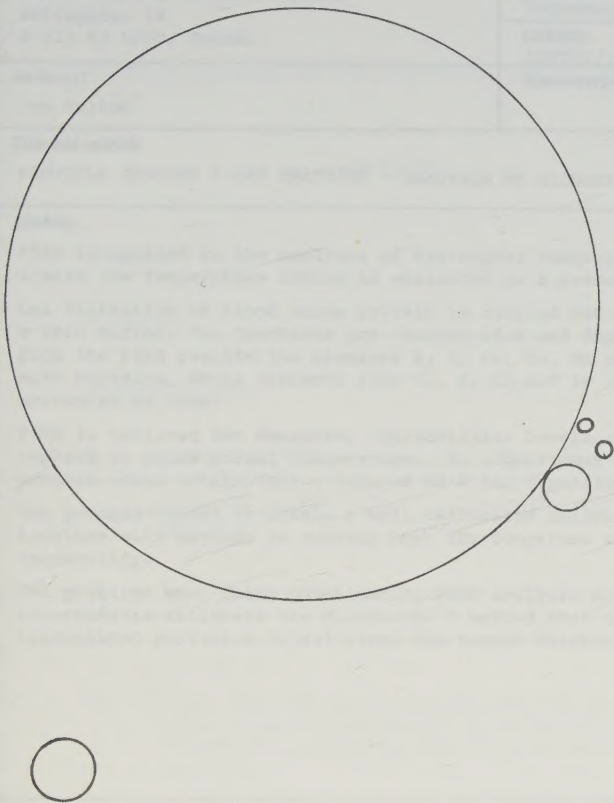
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Jan Pallon

DEPARTMENT OF NUCLEAR PHYSICS, LUND INSTITUTE OF TECHNOLOGY, 1986

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PARTICLE INDUCED X-RAY EMISSION - ANALYSIS OF BIOLOGICAL SAMPLES

Abstract

PIXE is applied to the analysis of biological samples. To improve the detection limits low temperature ashing is evaluated as a preconcentration method.

Gel filtration of blood serum protein is carried out with a Sephadex G-200 gel and a TRIS buffer. The fractions are concentrated and deposited onto thin backing foils. From the PIXE results the elements P, S, Fe, Cu, Zn and Se are found in connection with proteins, while elements like Cl, K, Ca and Br appears in connection to small molecules or ions.

PIXE is utilized for measuring intracellular levels of ions in human lymphocytes exposed to supra normal temperatures. No significant effects of this is found on protein-bound metals, but a reduced Na-K ion capacity is observed.

The process needed to obtain a well calibrated analytical PIXE set-up is described together with methods to control both the long-term stability and the day-to-day variability.

The problems when doing quantitative PIXE analysis on samples with an irregular intermediate thickness are discussed. A method that uses the energy loss of transmitted particles to calculate the target thickness distribution is given.

Key words

trace elements, protein separation, metal-binding proteins. PIXE, human serum, calibration, pre-concentration, ashing, multi-elemental, lymphocytes, hyperthermia, energy-loss, Rutherford scattering, intermediate thickness

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to my family

Particle Induced X-ray Emission - Analysis of Biological Samples

by

Jan Pallon

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Summary

Introduction

PIXE is a fast reliable technique for multielemental analysis of many small samples, which makes investigations of a large number of samples viable. The combination of biological sampling and PIXE has great potential in the fields of occupational and environmental health, as well as in medical, biomedical and clinical research.

Many people working in industry are victims of exposure to unknown amounts of pollutants of unknown composition. It is of fundamental importance to measure and control such exposure.

Biological monitoring - relating changes in biological specimens to a certain exposure to pollutants - is a complementary method to environmental monitoring in which the exposure is estimated by measuring pollutants in air, food, water etc.

Samples possible for biological monitoring are blood, urine, sweat, hairs, nails, etc. In very special cases biopsies of e.g. bone can be used. However, care must be taken to ensure that, for the sample type chosen, an observed elemental change has a biological relevance.

The PIXE method

Particle Induced X-ray Emission (PIXE) is a fast, sensitive, multielemental technique capable of quantifying elements heavier than aluminum. The absolute detection limits are in the ng range, and are achieved after an irradiation time of a few minutes. With PIXE it is possible to analyse samples in the range of μg to g

The method was first developed in Lund in 1970 by Johansson, Akselsson and Johansson [1]. Since then, the number of laboratories around the world that uses PIXE has grown, and by now number several hundred. There have been four international conferences on PIXE; in Lund in 1976 and 1980, in Heidelberg in 1983, and in Tallahassee, Florida in 1986 [2-5].

The principle of PIXE is that accelerated charged particles (protons, alphaparticles, deuterons) with an energy of a few MeV/u bombard the target. Vacancies are produced in the inner shells of the target atoms. These are filled by electrons from the outer shells and X-rays may simultaneously be emitted. The energies of the X-rays are characteristic of the atoms emitting them. By measuring the energy and intensity of the X-rays, quantitative analysis can be performed.

If the target is thin enough to cause no change in energy of the charged particle when passing through it, quantification is quite straightforward. On the other hand, if the target is not sufficient thin, the charged particles will be slowed down and the corresponding X-ray production cross section changes. X-rays emitted from deeper parts of the sample will suffer from absorption. Together this will lead to an underestimation of elemental contents. In a thick target the beam is completely stopped, and if the main elemental composition of the target is known, the effects of particle slowing down and absorption can easily be corrected for.

The analysis is carried out in a special irradiation chamber, where the beam, after having passed through a set of collimators, hits the target. Some of the emitted X-rays emerge through a thin foil and are detected in a Si(Li)-detector outside the chamber. The irradiation can take place either in vacuum or in an atmosphere of e.g. helium.

Biological samples

To meet the analytical requirements, most of the biological samples have to be pretreated so that they can withstand the charged particle bombardment. The pretreatment involves the removal of water which, at the same time, serves as a preconcentration step. Although the absolute sensitivity of PIXE is very high, the relative sensitivity is not high enough to allow detection of many trace elements in biological matter at their level of natural occurrence. Thus, the development and evaluation of suitable preconcentration and sample preparation methods has been essential.

The requirements placed on methods of pretreatment are that they should take advantage of the features of the PIXE technique and that the pretreatment should change or destroy the elemental information in the original samples as little as possible. A suitable method is

freeze-drying which is sometimes combined with low temperature ashing (paper I). Targets are prepared either by spotting solutions onto thin foils, or if enough material is present, by pressing the material into pellets.

In many cases the preconcentration achieved using these traditional methods is insufficient, or the presence of one or a few elements in the matrix masks the other elements. One example of this is the high content of alkali metals in urine which complicates the determination of trace elements, another is the presence of sodium in biological matrices which gives rise to a disturbing background from Compton-scattered γ -rays. Specific separation is then required. An example of this is the use of ion-exchange chromatography to selectively preconcentrate urine [6]. Another example is given in papers III and IV, in which gel filtration is used to separate blood serum proteins. The elemental content of proteins and macromolecules is measured and related to their size. Studying elemental content or the change in content in specific proteins yields much more biologically relevant information than studying the blood as a whole.

The separation method used here - gel filtration - is not the ultimate separation method, but was found to be suitable when trying to combine the requirements of protein separation at trace element level with the analytical requirements of PIXE. Gel filtration is not the only method that can be used for separating blood serum proteins, other methods may be used depending on the purpose of the analysis. The important point is the ability of the method to meet the requirements of PIXE and to take advantage of its capabilities.

A point of major importance is the analytical quality. The accuracy of the results from biological samples depends on the same general properties of PIXE as the accuracy in results from other types of samples. Important features, which are discussed in paper II, are the basic system calibration, the long-term stability and the day-to-day variability.

In the case of biological samples other aspects of analytical quality also become important. The elements of interest are present in very low concentrations. The analysis procedure from the original biological sample to the analysed target involves different stages of preconcentration and preparation. It must be born in mind that during

all of these phases there is a risk of loosing elements or parts of the sample, or of contamination. The only way to guard against such occurrences is to always be very careful and to critically investigate the different stages involved.

As an illustration, the concentration detection limits of PIXE are below 10^{-6} in the irradiated target, and a typical ratio of dry weight to wet weight for a body fluid is 0.07. The corresponding wet weight concentration detection limit for PIXE will therefore be around $7 \cdot 10^{-8}$ g/g. An upper limit on the acceptable level of contamination in handling a sample will, in this case, be of the order of 10^{-8} . All work must be performed in a clean environment with equipment cleaned and kept at trace element level. The use of at least duplicate samples and blanks is necessary, and the use of internal standards added at an early stage is strongly recommended. The internal standard makes it possible to check on the overall recovery, but will not reveal a contamination.

An important point in quantitative analysis is the thickness of the target, which must either be thin or thick (see above) - or be known. Many samples of biological origin will not fulfil this criterion; a common way of preparing the final target involves spotting a solution onto a thin foil. If special care is not taken, thick structures such as rings and crystals, can form, leading to the risk of underestimating the elemental content in the sample. The use of an internal standard can prevent this by indicating a low recovery. In paper VI the problems concerned with thick targets and the measurement of sample thickness are discussed in more detail.

Thesis

The aim of this work presented in this thesis was to apply the PIXE technique in the biological and medical field. The aims can be summarized in the following points.

a) To develop and investigate preparation techniques that allow high quality PIXE analysis to be performed on biological/medical samples.

b) To apply the technique to samples resulting from biochemical separation, and thereby demonstrate the potential of a combination of a speciation method with the multielemental capabilities of PIXE analysis of small samples.

c) To increase the capability of PIXE by making the accurate quantification of samples of intermediate thickness feasible.

Paper I

The use of the preconcentration method low temperature ashing (LTA) for ashing the organic compounds of a biological sample was investigated. The purpose of ashing is to reduce the mass of the matrix thus increasing the relative concentration of non organic compounds.

The conclusion arrived at was that for many biological samples LTA does not increase the sensitivity by more than a factor of 5 - 10, depending on the type of target. There is also a risk of losing volatile elements or of contaminating the sample; a problem which also is discussed in the paper.

Paper II

One of the advantages of PIXE, compared with, many other analytical methods, is its ability to act as an absolute quantitative method. Once the analytical set-up is calibrated, it can be used for measurements on samples with completely unknown elemental composition without the need of simultaneous analyses of standards. To be able to do this, a rigorous calibration process is needed, which is described

in this paper. For the calibration commercially available standards were used. The parameters of a physical model were adjusted to fit a calibration curve. The accuracy of the calibrated system in Lund is estimated to be better than 5% for individual elements.

Another important feature is the long term stability of the set-up and ways of achieving and monitoring it. By the irradiation of a target consisting of a thick silver plate with a small copper spot, sensitive and fast checks are performed.

Paper III

This paper focuses on the possibility of analysing many and small samples. Here, PIXE is used for determining elemental compositions in blood serum proteins. Various protein separation methods were first investigated, e.g. electrophoresis and isoelectric focussing, but these methods were either found to be too insensitive or impossible to combine with the requirements of trace element analysis. Instead gel filtration was chosen.

This paper also illustrates the problems of adopting preparative methods from other disciplines - here biochemistry - for use with PIXE. The first natural step was to use the standard biochemical method, at trace elemental level, and perform the experiments. It turned out however, that the chemical buffer, which was used in the separation process, contained a relatively large amount of chlorine which seriously interfered with the PIXE analysis. The consequence was high background in the spectrum allowing the determination of major elements only. Two ways around this problem were tried, low temperature ashing of the separated protein fractions, and changing to another buffer not containing chlorine. The use of LTA led to a dramatic increase in the sensitivity but at the same time the introduction of some contamination. Using a non-chlorine buffer proved to be succesful in lowering the detection limits, although in this case, contamination at some early experimental stage resulted in poor data.

Paper IV

The method of gel filtration was further explored in this study, and a standard technique was developed. To remove possible metal

contamination from the buffer, an ion exchanger was placed between the buffer supply and the separation column. The targets were prepared as "sandwiches" between two thin foils, thus allowing a proton beam of 70 nA to be used for the analysis. Five separations of serum from the same origin were performed showing reproducible elemental distributions for various proteins. The elements Fe, Cu, Zn and Se were found among the protein peaks, and sulphur and phosphorus were shown to be closely related to the protein content. S also appears close to the peak of small molecules together with Cl, K, Ca and Br. For the major peaks in the elemental distribution, peak-to-peak ratios agree within 10% for the five separations. Such elements are P, S, Cl, K, Ca and Br. In order to improve the separation reproducibility further, it is necessary to have high-quality separation equipment. To be able to study elemental binding to specific proteins a combination of gel filtration and a high-resolution separation method is required.

Paper V

Trace elements are of vital importance for biological processes. Both increased and reduced levels are known to have serious consequences for cellular life. Knowledge about intracellular levels of ions is of great interest for the understanding of many biological processes. PIXE is utilized for measuring intracellular levels of ions in human lymphocytes exposed to supra normal temperatures. Samples of lymphocytes obtained from the same blood donor were analysed with PIXE and atomic absorption spectrometry and the results compared. No significant effects of supra normal temperature on protein-bound metals was found, but a reduced Na-K ion capacity was observed.

Paper VI

This paper describes the problems related to quantitative PIXE analysis of samples of an intermediate thickness. Various methods of determining the target thickness simultaneously with PIXE analysis are discussed. The method proposed in this paper uses the energy loss of particles (protons) that have passed through the sample which are then forward-scattered through a thin carbon foil and into a surface-barrier detector. From the pulse height spectrum it is possible to determine the variation in thickness of the target. The method is demonstrated on a sample with a step-wise increasing thickness. Finally, the errors resulting when thick-target corrections are made

assuming the sample to be of constant thickness, when in fact it has an irregular thickness distribution, are discussed.

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EVALUATION OF LOW TEMPERATURE ASHING OF BIOLOGICAL MATERIALS AS A PRECONCENTRATION METHOD FOR PIXE ANALYSIS

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Preconcentration is usually necessary to improve the detection limits of biological materials in PIXE analysis. Using two NBS standard materials – bovine liver and orchard leaves – the recovery of different elements after ashing is examined. Low temperature ashed blood plasma is compared with freeze-dried plasma to examine the improvement in the wet weight detection limits. Both thick pellets and thinner targets are analysed and a comparison is made.

1. Introduction

In this study the feasibility of low temperature ashing as a sample preparation technique for PIXE-analysis of biological samples has been tested for different matrices. The purpose of the ashing is to destruct the organic matrix, thereby concentrating the trace elements in the sample.

The traditional method of dry-ashing organic material is to use an oven in which the sample can be heated to temperatures of several hundred degrees centigrade. Due to the high temperature, volatile compounds or elements can be lost [1]. In the low temperature ashing technique [2,3], oxygen molecules in a gas stream are transformed into highly active oxygen atoms by using a high frequency electromagnetic field. The excited atoms react with the sample, converting primarily hydrogen, carbon and nitrogen into volatile oxides. Thus the matrix can be ashed without the use of a high temperature.

The probability of losing volatile elements during low temperature ashing is expected to be much smaller than in traditional ashing in an oven, but it cannot be neglected. The behavior of elements such as Hg, Br and Se is particularly difficult to predict. Another problem is the risk of sample contamination, which increases with the number of sample preparation steps. To check if such effects occur, well-known standard materials can be analysed. NBS reference materials have well-known compositions, which have been certified by the U.S. National Bureau of Standards [4], and determined by many independent analytical techniques. In the study reported here, two common NBS standard materials, bovine liver and

orchard leaves, were chosen.

The change in detection limits during ashing is a function of several factors, which give different results for different biological matrices. We chose blood plasma when studying the improvement in detection limits. Blood plasma is an interesting body fluid from the point-of-view of occupational health. The levels of trace elements in blood plasma from people working in industries could possibly be used to monitor their exposure to these elements. It is not possible to analyse the natural concentration for many elements of considerable interest using PIXE without preconcentration. Thus it is important to determine the experimental detection limits in blood-plasma before and after low temperature ashing.

2. Experimental

The NBS reference materials, originally freeze-dried and homogenized, were put into a glass crucible for ashing. The sample masses were determined before and after ashing by weighing. The blood plasma was first freeze-dried at liquid nitrogen temperature thus reducing its mass by a factor of 11. Next, 450 mg of dried blood plasma was low temperature ashed for 12 h yielding a mass reduction factor of 7.

Targets were prepared from both the freeze-dried matter and the ashed residue. Thick pellets were pressed from the freeze-dried parts of all three materials. From the ashed residues, thick targets were made from blood plasma and bovine liver but not from orchard leaves, due to lack of material. The thick pellets had diameters of 6 mm.

Targets were also prepared by dissolving the rest of the ashed residue in 0.5 ml of 7-molar supra-pure nitric acid and pipetting between 5 and 20 μ l on to Nuclepore filters (N 040, pore size 0.4 μ m, diameter 25 mm).

The targets were irradiated by 2.55 MeV protons from the 3 MV Pelletron accelerator in Lund [5]. In order to decrease the count rate from dominant elements such as S, K and Ca, an external pin-hole absorber was used. It consisted of a 340 μ m thick Mylar film with a small hole for the low energy X-rays to pass through. This absorber was chosen from our standard set of different absorbers as being the most suitable one. It would, however, have been better to use one with a ten times smaller hole.

The thick pellets were analysed for 300–500 s using a beam diameter of 4 mm giving a total accumulated charge of 3 μ C. The pipetted targets were analysed for 300–700 s using a beam diameter of 8 mm.

The total accumulated charge was 10 μ C. All irradiations were performed in a vacuum chamber and for some hot carbon filament was used to avoid charge build-up on the samples [6].

3. Results and discussion

3.1. Detection limits for blood plasma

From the detection limits in the sample, defined as three times the standard deviation of the background, the corresponding wet weight detection limits in the original blood plasma were calculated using known preconcentration factors. The results for the freeze-dried matter and for the low temperature ashed matter are shown in fig. 1. Also included is the natural occurrence of different elements in human blood plasma according to Iyengar et al. [7].

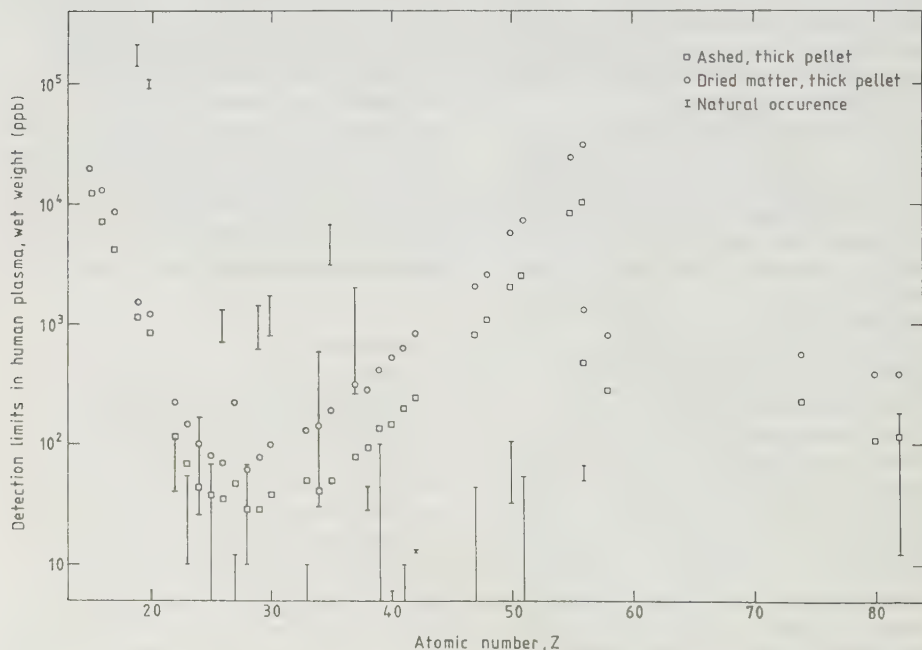


Fig. 1. A comparison of detection limits (three standard deviations of the background in the X-ray spectrum) in human blood plasma using different preparation methods. Freeze-dried samples are indicated with circles, low temperature ashed samples with squares. The vertical lines show the limits of the reported natural occurrence of trace elements in blood plasma as compiled by Iyengar et al. [7].

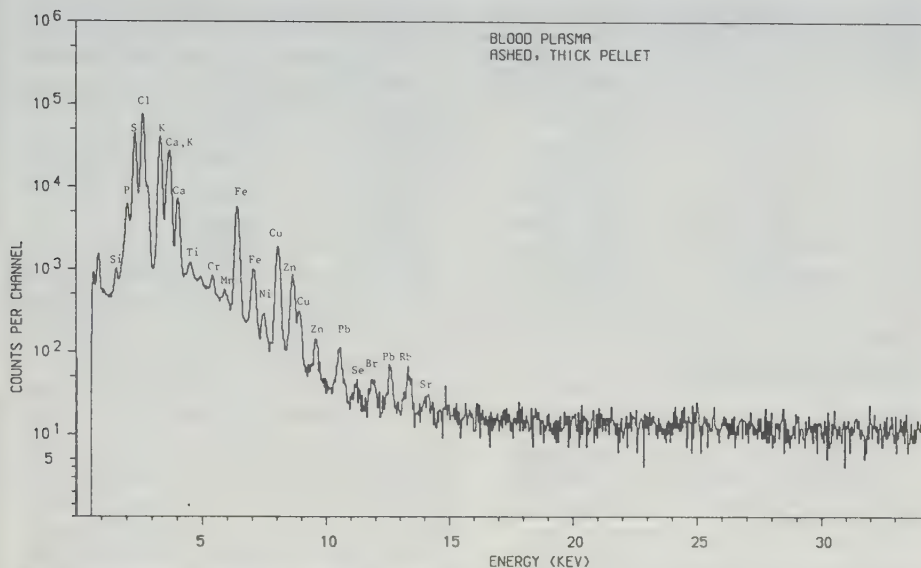


Fig. 2. Spectrum from an irradiated thick pellet of blood plasma. Analysis is made with 2.55 MeV protons during 350 s, accumulating $3 \mu\text{C}$. The larger background level at higher energies is probably due to gamma-rays from sodium in the sample.

The improvement effected by ashing samples rather than only freeze-drying them is obvious, the wet weight concentration detection limit being lowered by factors of 2–3. This increases the possibility of analysing elements such as Ti, V, Mn, Se and Pb in ordinary routine analyses. The mass reduction achieved in ashing blood plasma is about 7–8, but for heavier elements the gain is partially decreased due to a higher background. By comparison of spectra, the spectrum of the ashed blood plasma is found to have a generally higher background than the spectrum of the freeze-dried blood plasma. One explanation is that the ashing eliminates lighter elements such as H, C and O, thus giving the remaining matrix a higher average Z -value. Thus the sodium concentration, which in dried blood plasma is about 3.5%, has increased to about 25% after ashing. During proton bombardment sodium produces gamma-rays. Due to Compton scattering in the detector, these will give a significant contribution to the background. This gamma-ray background is rather constant over the whole energy region of interest for PIXE. Because of the decreasing sensi-

tivity of PIXE with increasing Z , the gamma-ray background becomes more important for heavier elements. As a result, the calculated concentration detection limits in the ashed *matrix* are higher than in the freeze-dried *matrix*. Despite this, due to the more concentrated matter, a number of elements which could not be seen in the freeze-dried sample are detected in the ashed spectrum (see fig. 2).

Since natural concentrations may vary, it is not always possible to determine the limit at which an analysis of a specific trace element will be possible. Clearly it is possible to determine more elements using low temperature ashing than only freeze-drying. In addition the detection limits are always a function of the irradiation time and the X-ray absorber used. With an optimum choice of these parameters, the detection limits will be further improved.

3.2. Analytical results for NBS standards

The recovery of different elements in low temperature ashed samples was investigated for a couple of

Table 1

Ratios of measured element abundance in NBS bovine liver (SRM 1577) to the stated value in percent. Mean value ± 1 standard deviation. The NBS-value in brackets is not certified, but given for information only.

Element	Dried, thick ^{a)}	Ashed, dissolved ^{b)}	Ashed, thick ^{c)}	NBS
P	92.7 $\pm$ 2.7	63.6 $\pm$ 3.6	116 $\pm$ 9	(100)
Cl	122 $\pm$ 4	<0.8	<0.9	100
K	105 $\pm$ 6	88.7 $\pm$ 4.1	127 $\pm$ 9	100 $\pm$ 6.2
Ca	145 $\pm$ 8	145 $\pm$ 12	169 $\pm$ 16	100
Mn	115 $\pm$ 4	99.0 $\pm$ 2.9	90.3 $\pm$ 7.8	100 $\pm$ 9.7
Fe	93.3 $\pm$ 5.6	89.6 $\pm$ 3.7	101 $\pm$ 8	100 $\pm$ 7.5
Cu	98.4 $\pm$ 5.2	88.1 $\pm$ 2.6	95.9 $\pm$ 7.8	100 $\pm$ 5.2
Zn	96.2 $\pm$ 4.6	92.3 $\pm$ 7.7	100 $\pm$ 8	100 $\pm$ 7.7
Se	100 $\pm$ 27	<82	<27	100 $\pm$ 9.1
Rb	98.4 $\pm$ 5.5	95.6 $\pm$ 5.5	<90	100 $\pm$ 5.5

a) 3 pellets.

b) 4 samples pipetted on Nuclepore filters.

c) 1 pellet, only analytical error stated.

standard samples. Tables 1 and 2 show the contents of the NBS standards bovine liver and orchard leaves, as found in the PIXE-analysis, as percentages of the values stated by NBS.

The left-hand column in table 1, named dried thick, gives the values for the original freeze-dried matter. Except for Ca these agree quite well with the

certified values. We have no reason to believe that the high Ca-value is due to an error in calibration, especially since the measured values in table 2, orchard leaves, show good agreement with the certified values.

During ashing of bovine liver, more than 90% of the chlorine and part of the selenium present are lost. When calculating the contents of the pellet, thick target corrections due to proton slowing-down and X-ray attenuation must be used. The low-Z element values are especially sensitive to the matrix composition, which is not completely known in the ashed specimen. In table 3 the effect on the PIXE-results when changing the assumed proportions of two components in a matrix are illustrated. These changes have most influence on the values for neighbouring elements and become less important for distant elements.

When making corrections for the thick, freeze-dried pellet made from orchard leaves, a cellulose matrix was assumed. Sulphur and chlorine values are

Table 2

Ratios of measured element abundance in NBS orchard leaves (SRM 1571) to the stated value in percent. Mean value ± 1 standard deviation. NBS-values in brackets are not certified, but given for information only.

Element	Dried, thick ^{a)}	Ashed, dissolved ^{b)}	NBS
S	84.0 $\pm$ 10	65.8 $\pm$ 2.6	(100)
Cl	82.6 $\pm$ 14	58.0 $\pm$ 4.3	(100)
K	109 $\pm$ 3	100 $\pm$ 3	100 $\pm$ 2.0
Ca	100 $\pm$ 3	95.7 $\pm$ 4	100 $\pm$ 1.4
Mn	114 $\pm$ 5	100 $\pm$ 3	100 $\pm$ 4.4
Fe	110 $\pm$ 17	73.3 $\pm$ 3	100 $\pm$ 6.7
Cu	117 $\pm$ 42	100 $\pm$ 8	100 $\pm$ 8.3
Zn	96.0 $\pm$ 8	324 $\pm$ 16	100 $\pm$ 12
As	150 $\pm$ 40	160 $\pm$ 20	100 $\pm$ 20
Br	100 $\pm$ 10	50 $\pm$ 10	(100)
Rb	100 $\pm$ 8	100 $\pm$ 17	100 $\pm$ 8.3
Sr	83.8 $\pm$ 5	97.3 $\pm$ 11	(100)
Pb	133 $\pm$ 22	122 $\pm$ 18	100 $\pm$ 6.7

a) 3 samples, cellulose matrix has been assumed.

b) 5 samples pipetted on to Nuclepore filters. Values have been obtained by assuming Mn to be accurate and normalizing the other elements to Mn (the original recovery was only about 70%)

Table 3

Percentage change in the PIXE-results if the assumed matrix composition is changed from 15% N, 15% Na (+25% P, 35% K+...) to (a) 10% N, 20% Na; (b) 20% N, 10% Na.

Case	Percentage change					
	P	S	Cl	K	Ca	As
a	7.79	3.65	3.45	3.01	1.38	-0.36
b	-7.78	-3.64	-3.43	-2.99	-1.38	0.36

low, but on the other hand, the NBS values are not certified, being given for information only. The values for As and Pb are rather high, but good agreement is found for Rb and Br. In the case of ashing, losses of S, Cl and Br seem to occur, while K, Ca, Cu, Rb and Sr are well preserved. The high value of Zn may be due to contamination in the dissolving and pipetting sequence.

From the results in tables 1 and 2, one can conclude that volatile elements are lost to some degree, but that most of elements are well preserved after low temperature ashing. If enough ashed material is available and it is possible to make a good estimate of the major constituents of the ash matrix, thick pellets are to be preferred. This approach has several advantages:

1) Easier target handling, including fewer preparation steps and minimum requirements of chemical treatment, decreasing the risk of contamination.

2) The sensitivity (pulses/(ppm $\cdot$ μ C)) is often higher for thick targets, but the uncertainties when calculating the corrections are larger especially for lighter elements.

3) Corrections made to thick target data can be based on the fact that the matrix is infinitely thick for protons and that absorption occurs for all X-ray energies. Pipetted samples can in general not be regarded as thin targets with negligible X-ray absorption. When the pipetted ash solution dries on the thin filter, crystalline structures may result. These crystals can be too thick, thus making the self-absorption of low energy X-rays and slowing-down of protons severe problems. To avoid this, pipetted solutions have to be diluted, or a tedious absorption control of each target made. The first alternative decreases the sensitivity, the second is time consuming: neither is very attractive.

As already mentioned, the mass reduction factor between freeze-dried and low temperature ashed blood plasma is about 7. However, for most human

organs and body fluids, the reduction factor seems to be in the range 20–25 [7,8]. In these cases low temperature ashing will produce a more significant decrease in the detection limits.

4. Conclusions

This study demonstrates that low temperature ashing is a useful technique for the preparation of biological samples. It has some advantages, e.g., lowered detection limits due to higher concentrations of trace elements. These limits are not always lowered as much as might be expected, since some elements could play a dominant role in the production of background radiation. Disadvantages, e.g., losses of elements, could be reduced by optimizing the ashing parameters. A reduction of the input power during ashing will decrease the effective temperature, as would some type of cooling system. It might also be possible to apply some type of chemical treatment so as to convert some of the elements into compounds of greater thermal stability, thus reducing the losses.

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CALIBRATION AND LONG-TERM STABILITY OF A PIXE SET-UP

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A general mass calibration procedure of a PIXE set-up is described. As an example, the results of the calibration of the PIXE set-up in Lund are given. For the calibration commercially available standards were used. The parameters of a physical model were adjusted to fit a calibration curve. To decrease errors due to inhomogeneities of the standard foils, the average from four different pieces of each foil was taken. The accuracy of the calibrated system in Lund is estimated to be better than 5% for individual elements and still less for ratios of adjacent elements.

For long-term quality control of the results, a special sample has been designed. This consists of a thick silver plate with a small copper spot in the centre. It is very sensitive to small changes in a PIXE set-up. By analysing this sample periodically, mistakes in the arrangement are promptly detected—a feature which is much appreciated in a mixed research, development and routine analysis laboratory. Also a measure of the long-term stability is obtained. For the Lund system, the long-term stability is 2.5% for a homogeneous sample and 4% for a small (inhomogeneous) sample.

1. Introduction

One important advantage of the PIXE-method compared to many analytical methods is the possibility of quantitative analysis without using standards. It is, however, initially necessary to establish a reliable calibration of the analytical system. This calibration may be performed in different ways. One approach is to procure accurate values of all the individual physical parameters of the system. Another way is to determine a sensitivity factor for each element of interest by fitting a calibration curve to experimental values. The second calibration procedure probably gives more accurate results than the first one if many good accurate standard foils are used. The second procedure is also less time-consuming.

In the Lund PIXE laboratory, we use mainly a calibration procedure suggested by Akselson et al. [1]. In this procedure, we try to minimize the uncertainty of the calibration by using a physical model to guide the fitting procedure in the second method.

To maintain high analytical quality, it is important not only to have an initially accurate calibration but also to secure good stability of the experimental arrangement. This can be made by periodically analysing samples sensitive to any significant changes in the system.

2. The PIXE arrangement in Lund

Since the Lund PIXE set-up will be used as an example in the following discussions, a short description of the set-up will be given. For routine experiments, 2.55 MeV protons from a 3 MV Tandem accelerator (NEC 3 UDH) are used. For maximum flexibility in the set-up a large irradiation chamber was constructed (fig. 1). The chamber was used both for routine analysis and for developments of the PIXE-method. One disadvantage of a spacious chamber, however, is the more complicated charge integration required. In our system, we integrate the beam current both on the chamber itself and in a Faraday cup. To get an accurate charge integration, we use a secondary electron suppressor system, applying +30 V to the last collimator and –50 V to the electron shield between the sample and the last collimator (see fig. 1). For routine purposes, we recommend a smaller chamber.

A homogeneous beam is obtained by using a thin ($\sim 1 \text{ mg/cm}^2$) gold-foil to diffuse the beam, and the beam-diameter may be varied from 1 mm to 1 cm by external collimator control.

The X-rays produced are detected in an 80 mm² (collimated to an effective area of 30 mm²) Si(Li) Kevex detector with an energy resolution of 158 eV at 5.9 keV. Instead of an ordinary pile-up rejector, we use an on-demand beam deflection system giving a pile-up interval of 300–400 ns.

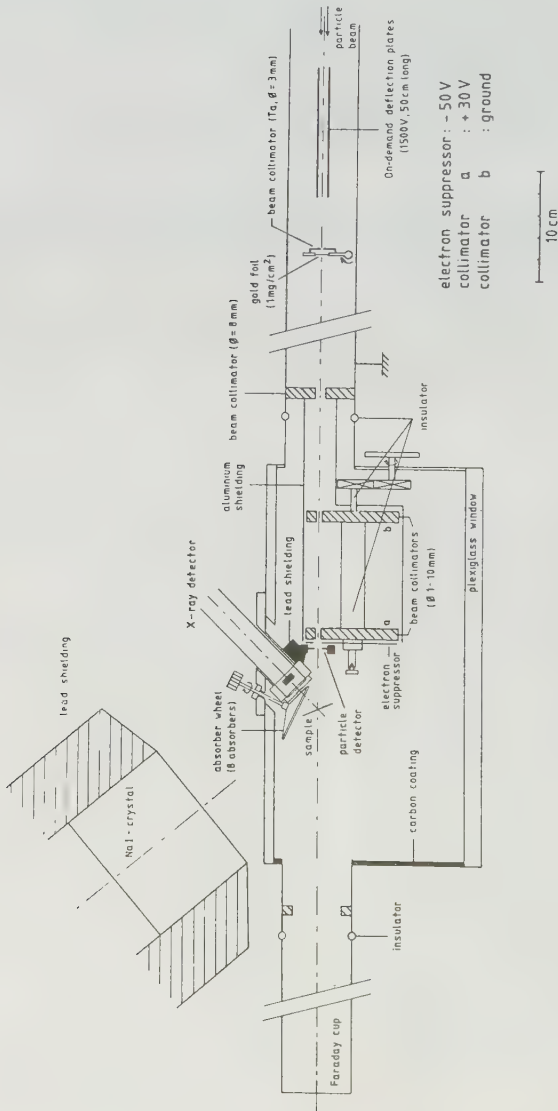


Fig. 1. The PIXE set-up in Lund.

A surface barrier ring detector to measure back-scattered particles and a NaI-crystal for gamma-ray detection are also included in the system (fig. 1).

To avoid charge build-up on thick insulating samples, a battery operated electron emitting carbon filament [2] is located close to the sample.

3. Calibration procedure

The possibility of access to a certain type of accelerator and also the kind of samples to be analysed determine the kind of charged particles selected for PIXE-analysis. In the PIXE set-up in Lund, we use mainly 2.55 MeV protons. The proton energy has been calibrated using the 1.374 MeV (p, γ)-resonance for fluorine, by irradiating a thin CaF₂-target evaporated on to a thick tantalum plate. The accuracy of the energy calibration is better than 10 keV.

3.1. Irradiation chamber performance

Before performing a mass calibration, it is necessary to test the linearity of the X-ray yield as a function of the count rate as well as of the accumulated charge. Fig. 2 shows the results of two such linearity tests of our system. Sample I has been irradiated using a moderate current (0.1–25 nA) giving count rates between 20 and 6000 c/s. For sample II, lower count rates (200–1300 c/s) and higher beam currents (10–65 nA) were used. No significant variation in the yield is found when the current is varied. When the count rate is increased a slight decrease in the

yield seems to occur. The difference between the maximum and minimum values is, however, only about 3%. To reduce pile-up peaks which could interfere with characteristic peaks in the spectra, most samples are analysed with a count rate below 2000 c/s. The maximum value of the current selected depends on the heat resistance of the sample irradiated and on the volatility of possible elements in the sample.

3.2. Calibration of thin, homogeneous samples

For the mass calibration, thin homogeneous and commercially available standards can be used. For our set-up, standards from MicroMatter Co were used [3]. These consist of a thin layer of the element of interest evaporated on to a Nuclepore filter. According to the manufacturer the accuracy is better than 5%.

The standards were analysed using a 0.53 cm² beam area and no external absorber except for the chamber window (Be). The count rates were all below 2000 c/s to reduce pile-up peaks in the spectra and the time of analysis was chosen to give a statistical error well below 1%. Altogether 45 different standard foils have been analysed for their K-lines. The stan-

Table 1

A list of the standard foils supplied by MicroMatter Co during 1977–1979. The standard compound is evaporated onto a Nuclepore filter.

Compound	Year of purchase	Compound	Year of purchase
Al	1977	As	1977
SiO	1979	CsBr	1977
GaP	1978	CsBr	1979
CuS	1977	RbNO ₃	1979
CuS	1979	SrF ₂	1977
CuS	1979	Y	1979
NaCl	1979	Nb ₂ O ₃	1979
KCl	1978	MoO ₃	1977
CaF ₂	1979	Ag	1979
Ti	1979	Ag-Hg	1979
V	1977	Cd	1978
Cr	1979	Sn	1977
Mn	1977	Sb	1978
Mn	1979	BaF ₂	1977
Fe	1978	BaF ₂	1979
Co	1979	WO ₃	1977
Ni	1977	Au	1978
Zn	1979	Pb	1979
		ThF ₄	1977
		UF ₄	1979

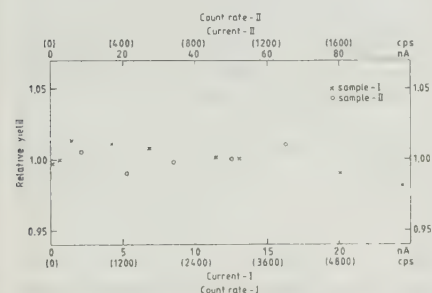


Fig. 2. Tests of the linearity of the X-ray yield (normalized to the mean) as a function of the beam current and the count rate for two different samples. The statistical error is less than 1%.

dards from the period 1977–1979 (see table 1) have been divided into four different pieces and all pieces have been analysed. The average value was used for the calibration.

A procedure often used in obtaining a calibration curve is to fit a nice-looking curve to the measured values and thus achieve sensitivity factors (counts/ $\mu\text{C}/\text{ng}$) for all elements [4]. We use a somewhat different approach in which the physical interpretation is stressed.

For our calibration, we use the formula:

$$A = c \cdot N / (P \cdot S \cdot T),$$

A = the mass per unit area of the element analysed (ng/cm^2),

c = a known constant ($0.00334 \times \text{atomic weight of the element}$),

N = the number of pulses in a peak per unit of charge [$(\mu\text{C})^{-1}$],

P = the X-ray production cross section in the peak (b),

S = the solid angle (sr),

T = the system efficiency.

To make a preliminary calibration, the X-ray production cross section is calculated from good results in the literature (e.g. from refs 5, 6 and 7). From simple measurements, the best possible estimates of the physical parameters of the analytical system (distance sample–detector, detector area, window thicknesses, thickness of gold and dead layer of the crystal, air gap and depth of the sensitive volume) are made.

Standards producing K-peaks of medium energy (4.5–17.5 keV) are used to determine the solid angle (S). The results for these elements are rather insensitive to small errors in the estimation of the system efficiency (T). We also check that the value of the solid angle determined is comparable within the uncertainties in the previous estimations of the distance detector–sample and of the area of the detector (total uncertainty <20%).

From results for lighter standard elements (1.5–4.5 keV), we determine the transmission through the system by making small changes of the used values of the window-thicknesses until we get by linear regression a horizontal line in fig. 3 in this energy region. By changing the value of the depth of the sensitive volume, the calibration for the heavier elements ($Z = 43$ –57) can be varied. Since the depth of the detector is about 5 mm, the system efficiency for the heavier elements is relatively independent of small variations in this value. A change in the depth of the sensitive

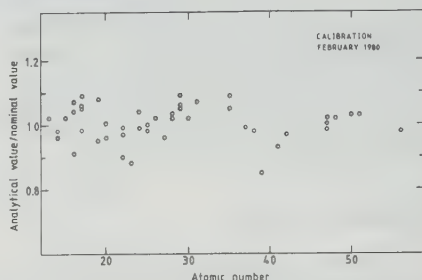


Fig. 3. The final results from the analyses of the standard foils divided by the values given by the manufacturer as a function of atomic number.

volume of the detector from 5 mm to 4.8 mm decreases the system efficiency by less than 1% for 30 keV X-rays.

Fig. 3 shows the results of the calibration after adjusting the physical parameters. The standard deviation of the mean is 0.8%. The following parameter values are used in our model: distance sample–detector 4.0 cm, detector area 0.28 cm^2 , chamber window $109 \mu\text{m}$ Be, air gap 0.4 cm, detector window $25.4 \mu\text{m}$ Be, gold layer $40 \mu\text{g}/\text{cm}^2$, dead layer $0.3 \mu\text{m}$ Si and depth of the detector 0.5 cm.

The manufacturer guarantees the stated thickness of each individual foil to be within 5% of its true value. The scatter of the results for adjacent elements as shown in fig. 3 indicates and further information from the manufacturer supports the supposition that any systematic component in the stated uncertainties is small. The error in the calibration due to uncertainties in the thicknesses of the standard foils is thus much smaller than 5%. Systematic errors in, e.g. cross section determination, charge integration and computer fitting of X-ray spectra all cancel out in the calibration procedure. If, however, any systematic errors occur only in part of the X-ray energy interval, they will remain. Such errors might be due to, e.g., local errors in cross section determination, incorrect estimation of the thickness of the detector gold layer or inhomogeneities in the detector crystal. The calibration curve in fig. 3 will give an estimate of the magnitude of this type of error. The accuracy of the calibration of a random element is estimated to be better than 5%. This estimation has been confirmed independently by Carlsson et al. [8], who used this calibration and thick target correction factors to obtain concentrations of several elements in some U.S. Geological Survey rock standards.

For heavier elements ($Z > 57$), the L-lines are used for mass calculation. To be able to calculate masses from different spectra containing L-lines using our fitting program [9], the relative intensities of the L-lines must be determined. This was performed by analysing thin single-element foils without any significant contaminations. By fitting the different peaks independently, their relative intensities can be determined.

The X-ray cross sections of the L-lines are not well-known and rather few elements have been analysed. Therefore a standard foil of each element of interest was analysed and the results used to determine a sensitivity factor for each element. The uncertainty of the calibration in this case depends directly on the accuracy of the single standard foil and due to outliers may exceed 5%.

3.3. Calibration of small thin samples

To carry out quantitative analyses using the PIXE-method, it is necessary to have either a homogeneous sample or a homogeneous beam. When small samples (smaller than the beam area) are analysed, it is important to use a homogeneous beam. In our system the homogeneity of the beam is obtained by letting the beam pass a small collimator (3 mm diameter) followed by a thin gold foil (about 1 mg/cm^2). The distance between the diffuser foil and the sample is about 55 cm. To check the homogeneity of the beam profile, a small Ni-sample (diameter $\sim 0.3 \text{ mm}$) is scanned step-wise over the beam cross section [10].

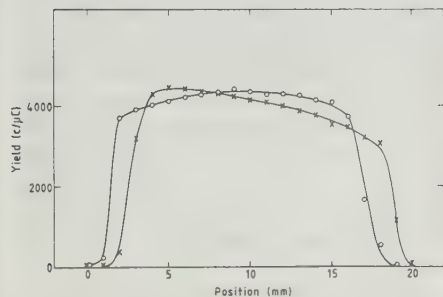


Fig. 4. The figure shows two vertical scans of the beam profile. A small (diameter $\sim 0.3 \text{ mm}$) Ni-sample has been used for the scans. In the first scan (o), the beam was on the cylinder axis of the accelerator tube (see fig. 1). In the second scan (x), the beam was about 3 mm off the axis.

Two vertical scans are shown in fig. 4. As can be seen from the figure, it is important that the undiffused beam hits the centre of the sample [11]. To guarantee this, the beam is collimated and steered so as to hit the sample centre. Without moving the beam, the gold foil is turned into the beam allowing the beam to pass the small collimator in front of it. The exact position of this collimator relative to the sample should then be maintained and is checked using micrometer gauges, horizontally and vertically. To attain increased homogeneity of the beam, it is possible to use a thicker gold foil or to increase the distance between the sample and the foil. Both these modifications will, however, decrease the beam intensity. The degree of beam homogeneity desired has to be weighed against beam intensity.

To be able to obtain quantitative results for a small sample from the mass calibration, the beam area has to be determined. By pipetting $5.00 \mu\text{l}$ of a standard solution (1.00 g of the element dissolved in 1.00 l of distilled water) on a Nuclepore filter, small and thin standard samples are prepared. The accuracy of an individual sample is better than 5%. From bombardment of these standard samples, the beam area for each collimator can be determined.

4. Homogeneity test of standard foils

The area of the standard foils used is about 11 cm^2 which should be compared with the area analysed (0.53 cm^2). If the standards are not homogeneous, this could introduce uncertainties into the calibration. When only a few standards are used for the calibration, this becomes especially important. All our standard foils, which were supplied by MicroMatter Co during the period 1977–1979 (see table 1), have been cut into four different pieces. All four pieces have one after the other been analysed with PIXE to check the homogeneity of the foils and, as mentioned above, to obtain a good estimate of their mean thicknesses. The beam current used when irradiating the standards has to be kept low to avoid losses of material or deterioration of the standard backing material. We normally used currents in the range $1\text{--}10 \text{ nA}$ and a beam area of 0.53 cm^2 . The maximum value of the proton current used on any standard foil was 20 nA . For the lighter elements (lighter than As), the irradiation time was typically around 100 s . An increase in the irradiation time was necessary for the heavier elements to get good statistics in

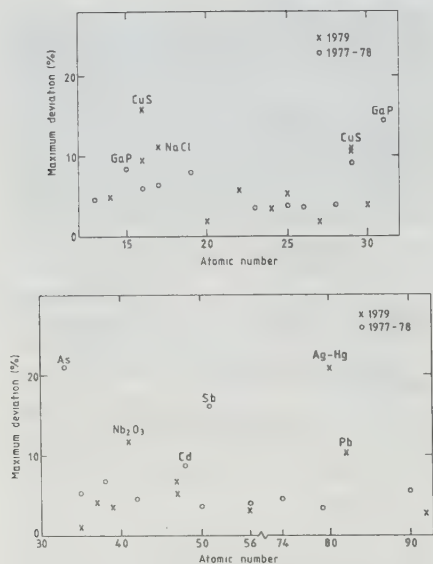


Fig. 5. The inhomogeneity of the different standard foils. The foils have been cut into four different pieces. In the figure is shown the maximum deviation (the maximum value minus the minimum value divided by the mean) in percent for different standards. For a single PIXE-analysis of a piece of a standard foil, the error due to statistics is less than 1%.

the peaks. When the same sample is analysed several times, the standard deviation is normally less than 1%.

In fig. 5 we have plotted the difference between the maximum and the minimum values of the separate pieces of a single standard foil as a percentage of the mean of all four pieces. For most of the foils, the deviation is in the range that might be expected but some outliers with a deviation of about 10% or more are present. The standard foils showing the largest deviations among their different pieces are GaP, CuS, NaCl, As, Nb₂O₃, Cd, Sb, Ag-Hg and Pb.

Using evaporation techniques when manufacturing standard foils may sometimes introduce a significant difference in thickness between non-adjacent areas of the foils. Since the thickness given by the manufacturer is an average value for the whole foil, our procedure of averaging the values obtained from the centre of each quarter of the foil is quite reliable. This statement is supported by the good agreement between the calibration values obtained in this way

(except for arsenic) from the less homogeneous standard foils and the values obtained from homogeneous standards of neighbouring elements.

However, there is another pitfall—the risk of losses of material due to the analysing technique used. Some of the parts of the standard foils used had been bombarded prior to this investigation which further accentuated this risk in our case. Repeated bombardments of foils revealed marked losses of arsenic and mercury (the silver of the Ag-Hg foil is not affected) in our experimental conditions. The extreme values for arsenic and mercury in fig. 5 may thus be explained by losses. The inhomogeneities of the NaCl, Nb₂O₃ and Pb foils are probably not accounted for by losses of material since the pieces of each foil which were earlier irradiated gave the highest values. The arsenic value was excluded from the calibration curve in fig. 3.

In other circumstances, the risk of loss of some elements other than As and Hg may constitute a problem, e.g., in calibration procedures using more damaging beams or using fewer different kinds of foils. Further investigations of losses from standards are currently in progress.

5. Quality control and long-term stability

It is very important for the users of all analytical methods to maintain good analytical quality. A well-calibrated system is not sufficient to guarantee this. Frequent checks of the stability and accuracy of the system must also be performed, e.g., by periodically analysing suitable samples.

For this purpose, a sample (or samples) which is quickly analysed and sensitive to significant changes of the analytical system should be used. It is also very important that simple errors due to operator oversight are rapidly detected. It is also important that the sample withstands all kinds of treatment without deteriorating, in order to make it possible to analyse the same sample over a long period of time. In our laboratory, MicroMatter standards (CuS) were originally used for quality control. We soon discovered that the standard backing material (Nuclepore) became fragile when irradiated for a period of time and that the standards were then easily damaged during handling.

The standard sample we have used for the last two years is made from a thick (1 mm) silver plate with a small piece (diameter ~1 mm) of copper foil in its centre. When analysing it, a beam current of 2 nA (count rate 2000 c/s) and a beam area of about 0.5

Table 2

The table shows probable explanations for different changes in the results from the analysis of the Ag/Cu-standard.

	Reason
<i>All peaks changed:</i>	Inaccurate charge integration. Detector badly positioned. Beam deflection system not working. Wrong secondary electron suppressor voltage.
<i>One peak changed:</i>	
Ag-L	X-ray transmission changed. Wrong angle between the sample and the detector.
Cu-K	Inhomogeneous beam. Collimator diameter not correct.
Ag-K	Wrong proton energy. Detector not working properly.

cm² are used and a 340 μ m Mylar foil is placed between the sample and the detector. To get a small statistical uncertainty (<1%) in the peaks of the spectrum, an irradiation time of 1 min is sufficient. The silver standard is analysed routinely before and after every batch of 40 samples and has so far been analysed more than 800 times.

In the spectrum from this Ag/Cu-standard, there are low energy peaks (Ag-L), medium energy peaks (Cu-K) and high energy peaks (Ag-K). In table 2, different patterns of changes in the areas of the peaks and their possible explanation are listed. When all peaks are changed by the same factor, it is often due to some error in the current integration. This error could be caused, e.g., by a leak current to or from the sample chamber or by not applying the correct voltage to the electron suppressors. Since a low current (2 nA) is used for the analysis of the sample, small variations in the charge integration can easily be detected. If the beam deflection system is not working, this gives a decrease of about 10% in all the peaks. Another reason for this kind of shift is a change in the solid angle, e.g., due to an incorrectly positioned detector.

When a variation mainly in the Ag-L peaks is detected, this is probably due to a change in the transmission of the X-rays. This kind of variation may also be due to a small change of the angle between the sample and the detector. A change of 5° from the normal angle of 22.5° gives a 7% error in the Ag-L peaks. The variation of the Ag-K peaks is then less than 1%.

If we find a change only of the Cu-K peaks, this is

probably due to beam inhomogeneity or because the beam area has been changed, e.g., by using the wrong collimator.

Finally, if the Ag-K peaks vary more than the other peaks, the reason may be that the proton energy is not correct. A proton energy of 2.65 MeV instead of 2.55 MeV gives an increased value for the Ag-K peaks of about 7%. Another explanation for the variation may be that the X-ray detector is not working properly.

We have also studied the long-term stability of our analytical system. The silver value (homogeneous sample) has a stability of 2.5% (= 1 standard deviation) over a one year period. The variation of the copper value for the same period was 4%. The larger variation for a small sample (spot) could be explained by changes in the homogeneity of the beam, as discussed earlier.

The variation over a long period should be compared with the variation when the sample is analysed several times during a short period (<3 h). The latter variation is less than 1%.

6. Conclusions

In the mass calibration procedure described, a fitting technique based on a suitable physical model is used. Another technique often used for calibration is direct fitting of a calibration curve to the measured values. One important advantage in using any fitting procedure is that single outliers of the results from the standard foils will have less impact on the calibration. Reasons for such outliers may be mistakes introduced during production or analysis of the standard foil, inhomogeneities of the foil or losses of standard material in the beam.

By using a physical model to guide the fitting procedure, errors due to incorrect standards in some part of the X-ray energy region can be further decreased. Especially when few standards are used, the direct fitting technique is very sensitive to the quality of the standard foils, and single outliers may introduce significant errors in the calibration. After having applied the calibration procedure described to our set-up, we estimate that an individual element can be analysed with an accuracy of better than 5% for elements with atomic numbers between 13 and 57 detected by their K-X-rays.

If only part of the available standard is analysed and rather few standards are used for the calibration, an additional error may be introduced due to inho-

mogeneities in the standards.

To maintain high quality in the analytical results, frequent controls of the calibration have to be made. A very versatile method for these tests of the stability, is the use of a thick silver plate with a copper spot. This sample is very sensitive to small changes in the analytical arrangement and withstands normal treatment without any noticeable deterioration. The long-term stability of the PIXE system in Lund is 2.5% for homogeneous samples and 4% for inhomogeneous. The short-term stability (<3 h) is better than 1%.

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PIXE ANALYSIS OF BLOOD SERUM PROTEINS, SEPARATED BY GEL FILTRATION

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Gel filtration of blood serum was carried out with Sephadex G-200 gel and tris buffer at pH 7.8. The amount of protein in each of about 100 collected fractions was monitored using UV absorption. The fractions were concentrated and deposited onto Kimfol backing foils.

From the PIXE results distributions of S, Fe, Cu and Zn were obtained in relation to the protein peaks, while elements like K, Ca and Br appeared at the position corresponding to small molecular sizes. Quantification, taking into account the intermediate thicknesses of the samples, gave reasonable values.

By adjusting the pH of the tris buffer with acetate instead of HCl, an increase in sensitivity was achieved, as the Cl is an important source of electron bremsstrahlung. By low temperature ashing still better detection limits can be reached, but at the expense of the loss of volatile elements.

1. Introduction

Many proteins in blood are involved in the transport and storage of elements. Some are very specific and bind the element tightly, e.g. ceruloplasmin binding Cu. A protein with a more general type of binding is albumin.

The detection limits with PIXE when analysing pellets made from freeze-dried serum are in the order of 70 $\mu\text{g}/\text{l}$ (wet weight). Detection of elemental concentrations below this value can be achieved if the elements are bound to specific proteins that can be isolated.

Human uptake due to, for example, industrial exposure may be more easily detected in specific proteins rather than in whole blood. To study specific proteins with PIXE, suitable protein separating techniques have to be found. The method must be non-contaminating, gentle to the proteins, e.g. not removing loosely bound trace elements, produce enough material to enable target preparation for PIXE and still have a resolution that makes the separation meaningful. The method investigated here is gel filtration.

2. Experimental

2.1. Principle

Gel filtration is a method where proteins are separated according to their sizes. The proteins, carried by

a suitable buffer, are forced to flow through a column packed with grains of an inert material. Proteins of different sizes penetrate into the pores of the grains to different degrees, and thus travel down the column at different rates, the largest travelling more quickly.

2.2. Experiment 1

A column with the following properties was used for the separation: bed height 1380 mm, internal diameter 55 mm, exclusion volume 800 ml, total volume 3200 ml, flow rate 12 ml/h. The gel was Sephadex G-200 (beaded cross-linked dextran, Pharmacia Fine Chemicals) and the buffer 0.02 M tris-HCl at pH 7.8. The separated fractions were collected with a fraction collector. A schematic drawing of the setup is given in fig. 1.

As the purpose of this experiment was to determine trace elements in serum proteins, all parts of the separation equipment had to be rigorously cleaned. Glassware and plastics were acid washed and the buffer was cleaned by ion exchange using Chelex 100 in ammonium form. The separation column and Sephadex gel were cleaned by taking two column volumes of 0.01 M $\text{Na}_2\text{-EDTA}$ through the packed column during a period of 15 days, followed by rinsing and conditioning with cleaned tris buffer for 13 days.

Ten ml serum from human blood, taken with a plastic cannula in order not to contaminate the blood with metal traces, was applied to the separation column. The separation, which was carried out at a temperature of $+4^\circ\text{C}$, took place during 9 days and resulted in 106 fractions each of 25 ml volume. From this, 0.5 ml was used for protein content monitoring by UV-absorption measurements at 280 nm. The fractions were freeze-

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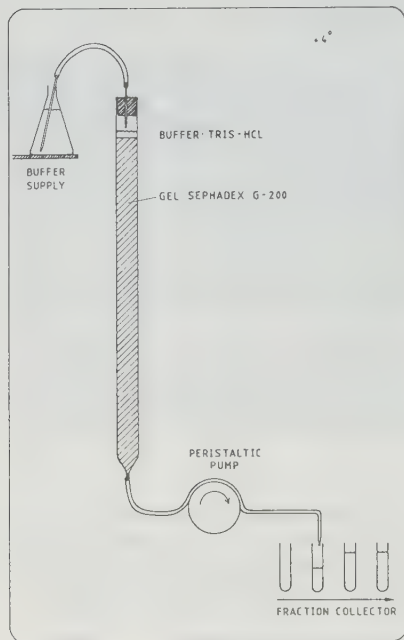


Fig 1. Schematic drawing of the setup used for gel filtration.

dried, dissolved in 500 μ l ultra-pure water (Milli-Q, Millipore), and 20 μ l of this was pipetted onto hydrophilised Kimfol foils. The targets were PIXE analysed with 2.55 MeV protons from the 3 MV tandem accelerator (NEC 3 UDH) at Lund, accumulating a charge of 20 μ C. To prevent charging an electron gun was used [1].

2.3. Experiment 2

A second gel filtration was carried out with a smaller column: bed height 935 mm, internal diameter 13 mm, exclusion volume 54 ml, total volume 128 ml and flow rate 1.4 ml/hour. The gel was Sephadex G-200 and the buffer 0.02 M tris-acetate at pH 7.7. Buffer, glassware and plastics were treated as in the first experiment. One ml serum of the same origin as above was used. Each fraction was collected for 1 h, and the whole separation took 100 h.

The fractions were freeze-dried, dissolved in 50 μ l of ultra-pure water and 20 μ l was deposited onto Kimfol foils. The PIXE irradiations were similar to those in experiment 1

2.4. Experiment 3

In experiment 1, only 1/25 of the volume of the fractions was used when preparing samples. In order to get a more efficient use of the material, the rest of fractions Nos. 1 to 52 were doped with yttrium, freeze-dried, low-temperature ashed, dissolved in 7 M HNO_3 and deposited onto Kimfol foils. PIXE analysis was performed as above.

3. Results and discussion

3.1. Experiment 1

Due to the high chlorine contents of the samples (the pH of the buffer was adjusted with HCl) a rather intense electron bremsstrahlung resulted. As a consequence, the detection limits for lighter elements did not become optimal. However, variations of S, K, Ca, Fe, Cu, Zn and Br with fraction number could be detected. Fig. 2 shows some elemental and protein content distribution curves.

The protein content curve shows three maxima in the first half of the distribution. The first (at fraction No. 17) contains, e.g. IgM and α_2 -macroglobulin, the second (at fraction No. 30) IgG, IgA and ceruloplasmin, and the third (at fraction No. 44) transferrin and albumin. The last peak in the distribution (at fraction No. 101) contains peptides, salts and different low molecular weight components.

When comparing the elemental distributions with the protein content curve, K, Ca and a major part of Br are found at the low molecular peak, indicating free ions and/or binding to small molecules. Fe has a marked maximum at the third protein peak and a less clear maximum at the first. Cu has a pronounced maximum at the second protein peak corresponding to the position of ceruloplasmin. Zn is seen at the third protein

Table 1

Trace element contents of blood serum in mg/l. (Exp. 1, sum of contents in all fractions. Pellet, thick sample made from the same serum. Ref. value, normal values (range, mean) as given in ref. 2.)

	Exp. 1	Pellet	Ref. value
S	658	758	(1200)
Cl	—	3820	2940–4120, 3600
K	157	164	160–211, 191
Ca	102	97	92–109, 97
Fe	1.34	1.78	0.87–1.87, 1.09
Cu	1.25	1.01	0.97–1.64, 1.19
Zn	0.47	1.98	0.67–1.83, 1.15
Se	—	0.08	0.098–0.327, 0.112
Br	2.69	3.23	1.04–7.5, 3.9

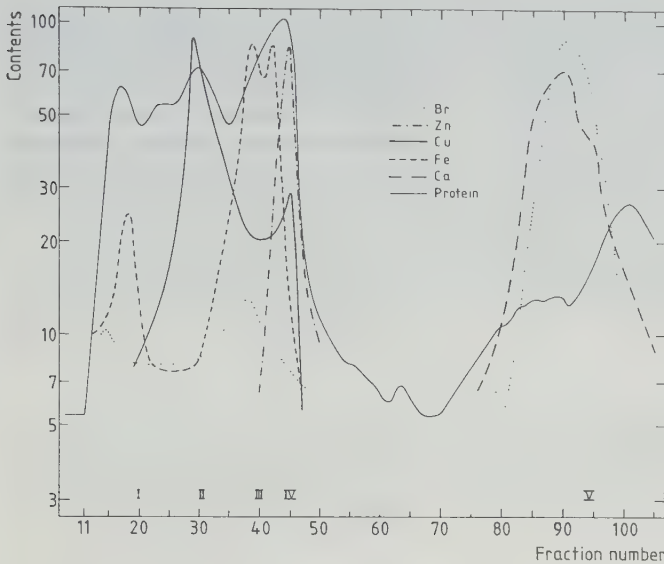


Fig 2. Elemental distribution versus fraction number after gel filtration of blood serum using a tris-HCl buffer. The content scale is logarithmic, the units arbitrary. The fraction number scale is roughly a logarithmic scale of molecular size with the smallest number corresponding to the largest molecular size. The roman numerals indicate regions used when calculating the amount to detection limit ratios given in table 2.

peak at a place a little distant from the Fe maximum.

By weighing the analysed, pipetted samples and measuring their dimensions, it was found that the samples had thicknesses ranging from 5.5 to 19 mg/cm². The estimated thicknesses were used in a computer code to correct for the effects of X-ray absorption and decreasing X-ray production cross sections on the results. The results of a summation of the amounts in the fractions are given in table 1. Most of the values seem reasonable when compared with the pellet made from the same serum and with the reference values. One value of exception is Zn, which is low in the gel filtration and high in the pellet. The low Zn value can be due to Zn lying below the detection limits in some part of the distribution, the high value due to some contamination in the preparation step of the pellet.

3.2. Experiment 2

With the use of a buffer not containing Cl, a clear improvement in the amount to detection limit ratio was made; see table 2. In contradiction to the observations made in experiment 1, the distributions of Cr, Ni, Cu and Zn here showed large peaks at the first protein peak. From the third protein peak the distributions were

similar to those found in experiment 1. This was taken as an indication of contamination of the separation system. Elements like Cl and P seem to have more reliable distributions, but due to the uncertainty of the system quality no quantification was carried out.

3.3. Experiment 3

These samples have still better amount to detection limit ratios, since they are prepared from almost whole fractions, and organic compounds together with Cl are

Table 2

The ratio of amount to detection limit in the three experiments (1) buffer tris-HCl, (2) buffer tris-acetate, (3) buffer tris-HCl and low-temperature ashed fractions. The regions I to V are shown in fig. 2. Missing data have the following explanations: n.d. not detected, (+) losses during ashing, (*) no sample made

Element Region	Ca I	Fe II	Cu III	S IV	Ca V
Exp. 1	n.d.	1.2	3.2	12	17
Exp. 2	4.8	3.8	7.4	223	34
Exp. 3	23	35	172	(+)	(*)

reduced during ashing; see table 2. Elements like Cr, Mn, Ti, V, Pb, Si and P are observed, but except for P, which shows a distribution pattern, they are thought to be background values originating from buffer and gel.

After normalisation to the yttrium values, the observed distributions were compared with those found in experiment 1. Due to experiments with dialysis and ultrafiltration, some of the fractions from the albumin peak were missing. The parts of the distributions that were complete agreed for the elements K, Ca, Fe, Cu and Zn with the distributions from experiment 1. Due to the missing fractions, the Zn and Fe distributions were incomplete, and quantification of their total concentration was not meaningful. Cu was nearly complete, and the amount found was 1.03 mg/l, which agrees with the values in table 1.

4. Conclusions

When combining gel filtration with PIXE it is important to: (a) use a buffer that gives a minimum of background in PIXE analysis (e.g. tris-acetate instead of tris-HCl), (b) clean (at trace element level) the buffer and separation system, (c) consider the actual thicknesses of the prepared targets.

Variations of S, Cl, K, Ca, Fe, Cu, Zn and Br with protein distributions can be detected after separation of 1 ml serum. Use of larger amounts of serum (demanding larger columns and longer separation times) does not directly imply better sensitivity in PIXE analysis. To achieve this low-temperature ashing can be used.

Serum contains numerous amounts of different proteins, which cannot be individually separated using only gel filtration. Combination with other separating techniques is necessary in order to find proteins with specific binding of elements.

We are grateful to Dr Ragnhild Cigén at the Department of Medical and Physiological Chemistry, Lund University, for valuable discussions and assistance which made this work possible.

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FEASIBILITY STUDY OF GEL FILTRATION AS A SEPARATION METHOD FOR BLOOD
SERUM PROTEINS IN COMBINATION WITH PIXE ANALYSIS.

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ABSTRACT

The combination between a protein separation technique and the PIXE method has a great potential for large surveys including thousands of samples, where multielemental analysis is required. Gel filtration with a Sephadex G-200 gel and a TRIS-acetate buffer was used for separating proteins in human serum. The fractions were doped with an yttrium/vanadium standard, then concentrated and pipetted onto Kimfol® backing foils. Using the PIXE technique the distributions of Fe, Cu, Zn and Se with respect to molecular size were found, indicating binding to specific proteins. S and P were found to correlate well with the protein content measured by UV-absorption at 280 nm. Further developments and tests on the protein separation technique is required taking restrictions imposed by the PIXE method into consideration.

Index entries

Trace elements, protein separation, metal-binding proteins, PIXE, work environmental health, human serum.

INTRODUCTION

The PIXE method is a fast reliable technique for multielemental analysis on small samples (1). Due to its low cost per sample, speed and accuracy, the method may make investigations including very large series of analyses realistic.

It is known that many proteins in blood are involved in the transport and storage of elements. Such proteins are of interest both from work environmental and clinical points of view. With the PIXE technique multielemental analysis can be performed with detection limits in the ng range in the analysed sample. PIXE is also well suited to the analysis of small samples (mg), and is thus capable of giving valuable information when combined with a suitable protein separation method.

The study of trace elements requires the separation method to be clean and also "gentle" to the proteins, not disturbing the original element binding. In earlier work (2) some experiments using gel filtration in combination with PIXE for the analysis of human serum were performed. This work is a further study on this subject. The separation system has been modified as well as the sample preparation technique. We have also checked the reproducibility of the separation process by investigating several samples of serum from the same batch.

METHODS

Principle of gel filtration

Gel filtration - or molecular sieve chromatography - is a separation process where molecules are fractionated due to their size. The molecules are forced to flow through a column packed with grains of an inert material. The smallest molecules are retarded during their passage through the column and come out at the end of the separation.

Apparatus

The separation column used had the following specifications: bed height 950 mm, internal diameter 13 mm, exclusion volume 43 ml and total volume 136 ml. The flow rate was 1.5 ml/hour. Tris-acetate with a molarity of 20 mM and a pH of 7.7 was used, together with a Sephadex G-200 gel (beaded cross-linked dextran, Pharmacia Fine Chemicals, Sweden).

Serum preparation

Human blood was obtained with a plastic cannula to avoid metallic contamination. A serum batch was prepared by homogenising the serum and dividing it into a number of 1 ml portions in separate plastic tubes. All tubes were then frozen and kept stored at a temperature of -32 °C. Serum was used instead of plasma because it could be obtained without adding anti-coagulating agents, which could be a source of contamination.

Cleaning

It was essential to carefully clean all parts of the separation

system. Thus all glassware was acid-washed in HNO_3 , while plastic tubes and connectors were washed in HCl .

The buffer was cleaned by passing it through an ion exchanger (Chelex-100 in ammonium form), which was incorporated into the separating system between the peristaltic pump and the separation column, see figure 1. This arrangement provides effective cleaning as any contamination is retained by the ion exchanger and the clean buffer flows immediately into the separation column.

The separation column and the gel were cleaned using 10 mM $\text{Na}_2\text{-EDTA}$ and then rinsed with clean buffer.

Separation and sample treatment

One ml of serum was applied to the column and separated during a period of 4 days. About 70 fractions (each containing 1.5 ml) were collected and were all treated in the following way: First 150 μl were withdrawn for protein monitoring using UV-absorption at 280 nm. Secondly, an elemental standard containing 300 ng yttrium and 300 ng vanadium was added. Then the fractions were freeze-dried, dissolved in 50 μl ultra-pure water (Milli-Q, Millipore) and 20 μl of this were pipetted onto Kimfol® foils. After drying, the samples were covered with a second Kimfol foil, thus forming a sandwich target.

Sandwich technique

The sandwich technique was introduced because in earlier experiments the samples sputtered during the proton bombardment. With the sandwich technique such problems are avoided, and it is also possible to carry out the analysis with a relatively high beam current ($\approx 70 \text{ nA}$).

Analysis

The targets were irradiated with 2.55 MeV protons from the 3 MV Pelletron tandem accelerator in Lund, accumulating a charge of 20 μC . The beam diameter was 7.9 mm. To prevent charging, a thin carbon foil was inserted in the beam close to the target to serve as an "electron shower". For all fractions except those containing the "salt peak" (see below), a 340 μm thick external Mylar® X-ray absorber with a small hole (0.9 % of the Si(Li) X-ray detector solid angle) was used.

RESULTS AND DISCUSSION

General

Typical results of the separation are shown in figure 2. It is seen that S and P correlate well with the protein content monitored by UV-absorption. Fe, Cu and Zn have peaks corresponding to specific proteins or groups of proteins. The first peak, which contains IgM and α_2 -macroglobulin, shows a small peak for Zn (and Cu). The second, containing IgG, IgA and ceruloplasmin shows a large Cu peak and also some Se. In the third peak, with transferrin and albumin, Fe and Zn are found.

At the end of the distribution elements appear in the form of ions or small molecules (the "salt" peak), here we find Cl, K, Ca, Br and some S and Fe.

The detection limits for the elements detected are given in table 1, and expressed in ng/fraction (≈ 1.5 ml).

Reproducibility

The results reported here are based on data from 7 separations, of which 5 are from the same serum batch. In all of these the elements show similar separation patterns. For the major peaks in the elemental distributions - in the five samples from the same batch - the peak-to-total ratios agree within 10 % for most of the elements detected. Such elements are P, S, Cl, K, Ca and Br.

For the elements Fe, Cu, Zn and Se the total amount found in the 5 separations from the same serum batch was calculated. The mean values and the relative standard deviations are shown in table 2 as well as the results from an analysis of a pellet made of freeze-dried serum from the same batch. Also included are some reference data from Iyengar et al. (3).

For the elements Fe, Cu and Se there is good agreement between different gel filtrations. Fe, Cu and Zn agree with the pellet data and the reference data. However only half of the Se is recovered in the gel filtration and it is detected in the protein peak region. The rest of the Se may be present in ionic form and eluted at the salt peak where the detection limits are increased, due to high contents of other elements, and hence not seen.

A major part of the Zn is found in the albumin peak and a little or none at all in the salt peak. However for some of the separations, Zn is removed from the albumin peak and smeared out on the fractions coming after, having a peak close to the salt peak.

A tentative explanation of this can be that small amounts of the $\text{Na}_2\text{-EDTA}$, due to insufficient rinsing with buffer, are bound to the gel until either free ions or proteins with easily removable ions pass. Then the metal binds to the EDTA which is then mobile and is transported through the column. We have used different ratios of volume of EDTA passed through the column to volume of buffer used for rinsing. Zn distributions for different EDTA-to-buffer volume ratios are shown in figure 3. It is seen that the larger the ratio, the more Zn is removed from the albumin peak. A ratio larger than 0.7 leaves nearly no Zn in the albumin peak. On the other hand, if no EDTA is used between consecutive separations, the Zn distribution is not affected at all.

Cu is rather strongly bound to ceruloplasmin, thus only a minor part is removed from the protein peak if EDTA is left in the gel. The Fe distribution seems not to be altered at all by the presence of EDTA.

Selenium

It is interesting to note that Se is detected in 6 out of 7 separations. In earlier experiments (not reported here) traces of selenium were found occasionally, but here it is observed in all 5

experiments performed on the same serum batch. Some reasons for this can be; a) higher proton charge per analysed target (lower detection limits), and b) the samples were covered with a second foil. It is possible that the sandwich technique makes the samples less sensitive to loss of volatile compounds of selenium.

CONCLUSIONS

It can be concluded that gel filtration is suitable for the separation of proteins to be studied using the PIXE technique for multielemental analysis. Apart from Fe, Cu and Zn, Se has been found among the protein peaks. S and P are closely related to the protein content and S also appears close to the small molecular peak together with Cl, K, Ca and Br.

Future plans include experiments on serum obtained from people exposed to metals in their working environment and comparing the results from serum taken from unexposed workers. A combination of gel filtration with a high-resolution separation method would also be of interest, as gel filtration alone has a limited resolution.

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TABLE 1

Typical detection limits with PIXE for some elements. The unit used is ng/fraction (≈ 1.5 ml).

Element	Det. limit
P	110
S	86
Cl	64
K	30
Ca	16
Fe	1.3
Cu	1.0
Zn	1.0
Se	0.94
Br	1.2

TABLE 2

Elemental concentrations ($\mu\text{g/L}$) in serum; mean values of five separations and corresponding relative standard deviations, values from freeze-dried serum from the same batch and reference values from Iyengar (3).

Element	mean value n=5	σ (%)	Pellet of dried serum	Iyengar
Fe	1400	9.3	910	1009
Cu	940	9.7	880	1190
Zn	960	16.	810	1150
Se	44	4.5	88	122

FIGURE CAPTIONS

1. A schematic drawing of the separation system. The gel used is Sephadex G-200 and the buffer is 20 mM TRIS-acetate with a pH of 7.7 (room temperature). To clean the buffer an ion exchanger containing Chelex-100 in ammonium form is included in the system.

2. Distributions of some elements in blood serum after protein separation by gel filtration. The content scales are logarithmic and the unit used is ng/fraction (a fraction $\approx$ 1.5 ml). The horizontal scale is roughly a logarithmic scale of molecular size. The curve A_{280} (in arbitrary units) shows the protein content monitored by UV-absorption at 280 nm. The peak at A contains IgM and α_2 -macroglobulin, that at B IgG and IgA, while at C mainly albumin is found. In the region after D come substances of low molecular weight.

Sulphur and phosphorus have distributions that correlate well with the protein content curve. Iron, copper and zinc have peaks at E, F, and G which coincide with the metal-binding proteins transferrin, ceruloplasmin and albumin, respectively. Se is found in the peak marked B. Potassium, calcium and bromine are found with the low-molecular-weight components at I, J and K.

3. The Zn concentration in different fractions for some values of the volume ratio of $\text{Na}_2\text{-EDTA}$ to rinsing buffer. All the Zn should be found at the albumin peak and none in the salt peak.

Symbol	$\text{Na}_2\text{-EDTA/Buffer}$ Ratio (v/v)
—	< 0.07
• • •	$\approx$ 0.1
- - -	$\approx$ 0.5
—	$\approx$ 0.7

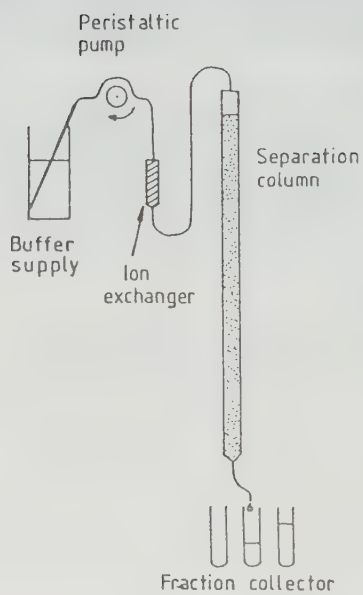


Fig. 1

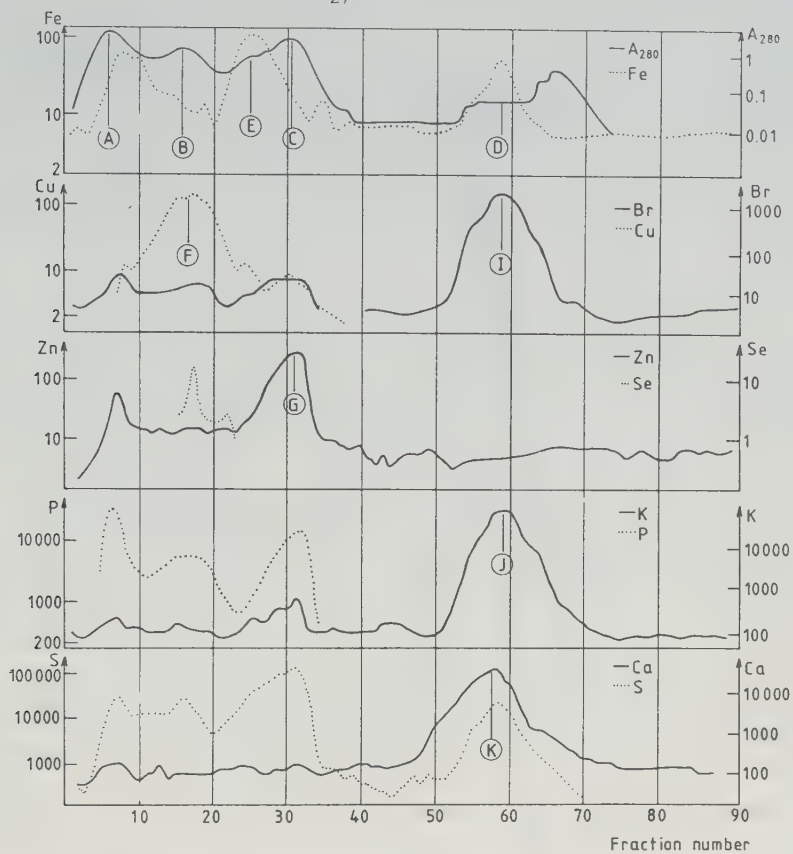


Fig. 2

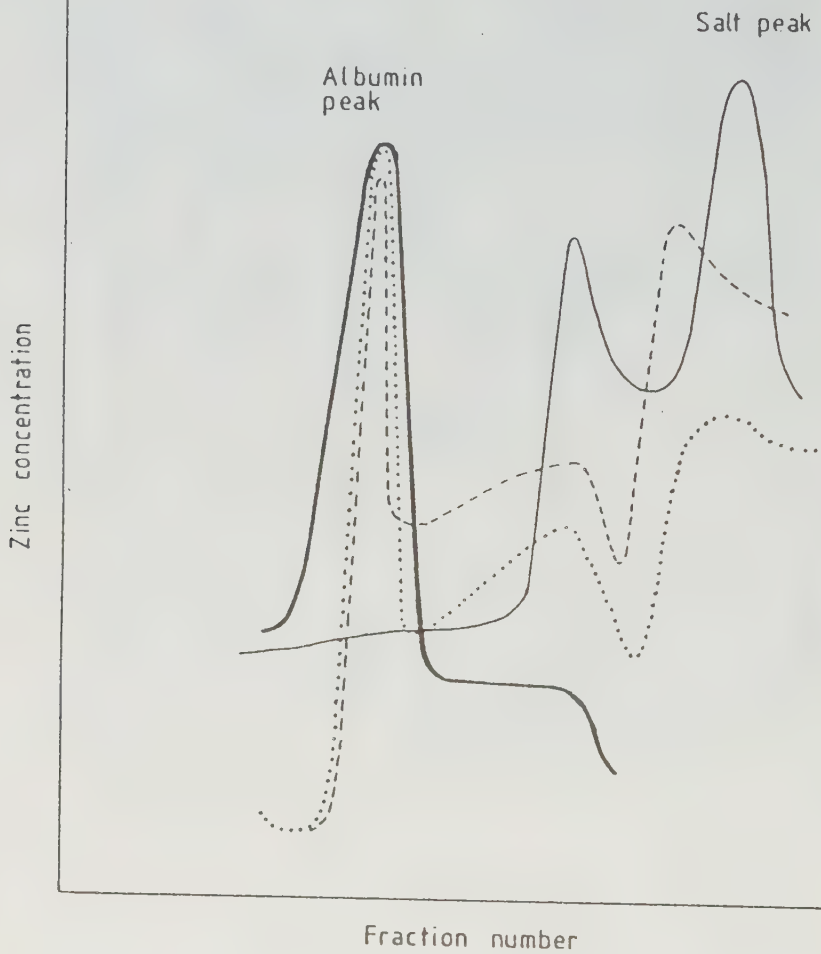


Fig. 3

UTILIZATION OF THE PIXE-METHOD FOR ELEMENTAL ANALYSIS IN HUMAN LYMPHOCYTES
AND ITS APPLICATION TO EFFECTS CAUSED BY HYPERTHERMIA¹

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ABSTRACT

The Particle-Induced X-ray Emission method (PIXE) is a fast and sensitive method for multi-element trace analysis. The PIXE method is described and its value for analysis of elements in biological samples is pointed out. The PIXE method and atomic absorption spectrometer are utilized for measuring intracellular levels of ions in human lymphocytes exposed to supra normal temperatures - hyperthermia. We find no significant effects of hyperthermia on protein-bound metals but observe a reduced Na-K ion pumping capacity. Possible backgrounds to this finding are discussed.

INTRODUCTION

Metal ions are of essential importance in biochemistry. The intra-cellular ion balance is crucial for maintaining osmotic pressure and electric potential across membranes. Some ions are required for protein synthesis and for activity of numerous enzymes. Other ions play a role in the transport of amino acids and sugars. There are also many rare ions, so called trace elements, of importance for biological processes (Prasad, 1976). Both reduced and increased levels of trace elements are known to cause serious consequences for cellular life, although the action of many of them are unknown.

Knowledge about intracellular levels of ions is of great interest for the understanding of biological processes following treatments with radiation, hyperthermia, antibiotics and other drugs, as well as in ecotoxicology.

One method for measuring many elements in one single experiment is the Particle-Induced X-ray Emission method - PIXE (for reviews see Johansson and Johansson, 1976, Khan and Crumpton, 1981 or Martin, 1984). The sample is irradiated with protons in the MeV range for excitation of the atoms. The de-excitation characteristic X-rays are then measured with a solid state detector. High sensitivity makes the PIXE method a suitable tool for routine multi-element trace analysis also for biological samples.

The aim of this paper is two-fold:

- (i) Comparison of the PIXE method with the more commonly employed method of atomic absorption for analysis of biological samples;
- (ii) Utilization of the PIXE method for measuring intra-cellular levels of ions in human lymphocytes after exposure of the cells to supra normal temperatures. The PIXE data are compared with similar data obtained from the same blood donors with an atomic absorption spectrometer.

Temperatures above the normal body temperature - hyperthermia - is known to induce many biological effects (Dietzel, 1975, Dewey et al, 1977, Field and Bleehen, 1979). Important targets for hyperthermia are the cell membranes (Wallach, 1978). Hyperthermia may affect lipid composition, fluidity and permeability of the membranes for various molecules and ions (Yatvin, 1977, Cress et al, 1978, Kwock et al, 1978, Yi, 1979, Yi et al, 1983a, 1983b).

We here report data obtained by the PIXE method and atomic absorption spectrometer, on intra-cellular contents of various ions following exposure of human lymphocytes to hyperthermia between 37 and 46°C. The two methods give comparable results, although the PIXE method is much more sensitive than the atomic absorption spectrometer. The data at control temperature (37°C) are comparable to those reported earlier in the literature.

At temperatures above 43°C the level of K decreases rapidly with increasing temperature with a simultaneous but smaller gain of Na. There is one earlier experiment by Yi et al (1983a) on mouse mastocytoma P 815 and HeLa cells also showing these effects. We find no significant effect of hyperthermia on protein-bound metals like Fe, Zn and Mg.

We suggest that the reduced efficiency of ion pumping observed could be related to the suppressed ATP production at high temperatures reported earlier by us for mononuclear leukocytes (Jonsson et al, 1984, 1985).

MATERIAL AND METHODS

Particle-Induced X-ray Emission (PIXE)

General

PIXE is a fast and sensitive method for determining elements in small samples (milligrams to grams) (Martin, 1984). The method can quantify elements heavier than Al simultaneously. The lowest detectable amount is in the order of parts of nanograms, while concentrations are measured at the ppm-level.

Principle

Protons (or heavier ions) with an energy of 2-3 MeV are produced by an accelerator. When the ions hit the sample characteristic X-rays are emitted. By measuring the radiation with an energy-dispersive X-ray detector - a Si(Li) detector - multielemental quantitative analysis can be performed. A routine analysis is carried out in 1-10 minutes.

PIXE is an analytical method similar to X-ray fluorescence (XRF), however, in that case the characteristic X-rays are induced by irradiation from a X-ray source. One advantage of PIXE compared to XRF is the possibility of analysing samples with very small dimensions. The proton beam can be focussed to micrometer dimensions and scanned across a sample in the same manner as with an electron microprobe. The sensitivity when using PIXE is often 1-100 times better than with electron microprobe, but the spatial resolution is not so good.

Set up

The proton beam was produced by the 3MeV Pelletron tandem accelerator at the Lund Institute of Technology. Samples were analysed in the PIXE routine chamber having an 8 mm diameter proton beam with an energy of 2.55

MeV (Fig. 1). The X-rays were measured with an Kevex 80 mm² Si(Li)-detector connected to a Nuclear Data ND6620-system and evaluated on a Norsk Data ND 100/500 computer system using the X-ray spectrum fitting/evaluating computer code HEX.

In this paper all samples analysed were prepared to be thin samples. This was done by pipetting 20 µl of the cellsuspensions onto Kimfol foils (a polycarbonate film, 180 µg/cm², Kimberly Clark).

Calibration and quantification

PIXE is an absolute method relying on such physical parameters as proton energy, X-ray cross-sections, detector efficiency, solid angle, and the X-ray transmission between sample and detector, in other words parameters that can be checked or controlled. Once the set-up is calibrated it can be used for determining amounts in unknown samples without the need of running elemental standards at the same time. However, to verify the correct condition of the PIXE set-up, a Ag-plate is analysed as first sample at the beginning of each batch of samples. With this check the quality of the analysis can be guaranteed.

Quantification is carried out by relating the number of pulses in a X-ray peak to the amount of protons that have caused the emission of the radiation. The amount of protons is determined by measuring the charge with a current integrator. The accumulated charge was typically 10 µC. The number of pulses/charge unit for a certain element, can directly be converted to a value of mass by using the calibration data. The precision and accuracy of the analysis is better than 10% if the number of pulses in the determined peak is larger than 10000.

Atomic absorption spectrometer

The analysis were carried out on a Varian Techtron instrument (Varian

Inc.) at the Department of Clinical Chemistry, University Hospital in Lund. Cells were lysed with 1% LaCl_3 in glass distilled water. LaCl_3 is a complexing agent which allows the release of Ca and Mg for accurate measurements. LaCl_3 was run as blank when analysing all elements. 150 μl of the suspension of lysed cells were injected into the spectrometer. Fuel was C_2H_2 with air as oxidant. Both lysates and blanks were diluted to ion concentrations in the linear range of the spectrometer calibration curves.

Isolation of lymphocytes

All glasswares used in these experiments were washed in 35% HNO_3 and then rinsed four times in distilled water which had been purified with ultrafiltration (Milli-Q-system, Millipore AB, Sweden). Physiological saline was prepared with ultrafiltrated water and NaCl (pro analysi, Merck).

Peripheral blood was taken by venous puncture into Vacutainers without any anti-coagulant from obviously healthy blood donors. The blood was carefully transferred to a glass flask and gently swirled with glass beads to defibrinate it and remove the platelets. The defibrinated blood was centrifuged at $100 \times g$ for 15 min and the serum removed. The blood cell fraction was diluted to original volume with physiological saline and layered on a Isopaque-Ficoll cushion (Lymphoprep, Nyegaard and Co.), which was centrifuged at $600 \times g$ for 20 min. The mononuclear leukocytes were collected from the interphase zone and washed three times in physiological saline. The number of cells were counted in a Coulter counter (model ZB, Coulter Electronics). Cell distribution analysis showed that about 98% of the mononuclear cells were lymphocytes.

In order to study if the number of cells on the Kimfol foil would affect the yield of ions measured, different amounts of cells were taken out from the cell suspension; 20 μl samples adjusted to contain $0.1 - 2 \cdot 10^6$ cells were applied onto the foils and dried in dust-free atmosphere.

Hyperthermia treatment

Cultures containing about $5 \cdot 10^6$ cells were prepared at room temperature in 4 ml of Hank's Eagle Medium plus 1 ml autologous serum. The culture medium was supplemented with 2.5 mM L-glutamine, penicillin (50 $\mu\text{g/ml}$) and streptomycin (50 $\mu\text{g/ml}$).

The cells were placed in water baths for 45 min at temperatures ranging from 37 up to 46°C. The temperature was measured with an accuracy of about $\pm 0.2^\circ\text{C}$. The heating period of 45 min was chosen mainly because that exposure time is used in the clinical application of hyperthermia as radiosensitizing agent in our hospital (Lindholm et al, 1982).

Immediately after the hyperthermia treatment the cell cultures were transferred to a 37°C incubator and maintained there for time periods up to 5 hrs to allow repair of damage.

After the appropriate incubation times the cells were spun down and washed three times in the physiological saline and 20 μl samples adjusted to contain about 10^6 cells were applied onto the Kimfol foils and dried in dust-free atmosphere.

RESULTS

Element analysis

A typical PIXE-spectrum of human lymphocytes is shown in Fig. 2. Effects on element yields of the number of cells on the Kimfol foils are shown in Fig. 3. Clearly there is good linearity within the cell density range studied ($0.1 - 2 \cdot 10^6$ cells/20 μ l).

Table I shows a comparison between results obtained with the PIXE method and the atomic absorption spectrometer for the same blood donor ($n=4$). The two methods obviously give comparable results, although the detection limits are considerably lower with the PIXE-method. There are in the literature unfortunately only few data reported on ion levels in human lymphocytes and mostly in units not directly comparable to ours. However, Bonnet et al (1984) report with PIXE method for Fe and Zn ($n=10$) $9.9 \cdot 10^{-3}$ μ g and $4.7 \cdot 10^{-3}$ μ g per 10^6 lymphocytes, which correspond to 0.2 and 0.07 fmol/cell respectively with a dispersion of 60%. Dennes et al (1961) measured with neutron activation method a Zn level of $14,0 \pm 1.9$ μ g/ 10^9 cells, corresponding to 0.21 ± 0.03 fmol/cell ($n=30$). They found significantly lower Zn yields in leukocytes from leukaemic subjects. Segel et al (1975) report with atomic absorption spectrometer for human lymphocytes ($n=15$) a K-level of 22.2 ± 4.4 fmol/cell and for Na 4.5 ± 1.9 fmol/cell. From data by Ryan et al (1980) we deduce a ratio K/Mg of 9.9 ± 0.5 ($n=20$); K measured by emission spectrophotometry and Mg by atomic absorption spectrometer, which can be compared to our ratio (atomic absorption spectrometer) 12.5 ± 2.9 .

It should be pointed out that there are big inter-individual variations of ionic levels, expected mainly to be due to nutrition and health status.

Hyperthermia effects on intracellular ion contents

The potassium level at 37°C (Fig. 4) remained fairly constant up to 5 hrs of maintenance in culture (i.e. within the experimental accuracy). At 42.5°C there is indication of a loss of K soon after treatment but the level returns to control level within about 1 h. At 44°C the K pool remains low for the whole time period studied (i.e. about 50% of control level). The insert in Fig. 4 is the temperature dependence of the K level after 5 hrs repair incubation at 37°C. For Na (Fig. 7) we note a slight gain with increasing temperature. At 46°C the K loss is about 75%, but the Na gain is only about 40% as measured 5 hrs after heat treatment. For all other ions measured; Zn (Fig. 5), Fe (Fig. 6), Mg, Ca, Mn, Cu, Br, and Rb (data not shown) we find no significant changes with temperature.

DISCUSSION

Element analysis with the PIXE method

In PIXE the analyses is restricted to a thin surface layer of the sample. The targets must be either thinner than this surface layer (thin samples, $\leq 2 \text{ mg/cm}^2$) or thicker (thick samples, $\geq 15 \text{ mg/cm}^2$). To get correct quantification in the case of thick targets the sample must be homogenous. With thick targets it is also possible to analyse Na, Mg, Li, F and B with a complementary method, Particle Induced Gamma-ray Emission (PIGE).

PIXE is multielemental with the maximum sensitivity for elements with atomic number 25-30, see Fig. 8.

However, due to the sample composition this sensitivity can not always be achieved:

1. Large amounts of an element causes the background to be higher in the vicinity of a peak; a special case is when the K_{β} X-ray peak of an element coincides with the K_{α} peak of another heavier element (Fe on Co, K on Ca, etc);
2. Large amounts of lighter elements give rise to a more intense X-ray production and thus a high count-rate at the analysis. Due to limitations in the detection system the count-rate must be kept below certain values. This is done by putting X-ray absorbers in front of the detector, but at the same time the sensitivity for lighter elements is reduced;
3. Some elements with high concentrations in biological matrices, e.g. Na, give rise to a general increase in the background due to the emission of γ -rays which are scattered in the X-ray detector. The reduction in sensitivity is most significant for elements heavier than As. The problem is most critical when analysing thick samples.

Hyperthermia effects on intracellular ion contents

We have found that the levels of K and Na remain relatively constant up to 43°C, above which the content of K decreases rapidly with increasing temperature (Fig. 4) with a simultaneous but smaller gain of Na (Fig. 7). Similar net changes of K and Na levels were observed by Yi (1979) and Yi et al (1983a) for P815 cells heated to 43°C. Ruifrok et al (1985) found that the K level decreased gradually with time in mouse fibroblasts heated to 44°C for 20 - 30 min. They also found a correlation between cell survival

and K content already at small loss of intracellular K.

For the protein-bound elements Zn (Fig. 5), Fe (Fig. 6), Mg, Mn, Cu, Br, and Rb (data not shown) there is no change in ion content with increasing temperature. Also the level of Ca as measured by atomic absorption spectrometer remained constant within the experimental accuracy.

The different ion currents are not expected to be independent of each other because of electric charge conservation. Due to high background levels from physiological saline we were not able to measure the level of Cl, but from work of Yi et al (1983a) one can expect that the K ion current is greater than the Cl ion current which means a very important influx of hydrogen ions. This influx of H^+ means lowering of intracellular pH which for a tumor tissue with its already high acidity could be lethal. From cell distribution analysis on the Coulter counter (see experimental details) we found no reduction of cell volume following hyperthermia treatments. Yi et al (1979) similarly found that the loss of cations did not induce a concomitant reduction, but rather a small increase in cell volume.

The mechanism behind the changes of K and Na contents could be due to less effective energy-dependent ion pumping. Hyperthermia also alters amino acid transport kinetics in a way suggesting that hyperthermia either reduces the number of carriers in the membrane or slows down the rate of transport across the membrane (Kwock et al, 1978). Furthermore, $Na^+ - K^+$ -ATPase activity, like many other enzymes, has been found to be temperature sensitive (Wisnieski et al, 1974, Burdon and Cutmore, 1982, Burdon et al, 1984), which in turn could affect ion pumping. However, other authors (Bates and Mackillop, 1985, Anderson and Hahn, 1985) have found the $Na^+ - K^+$ pump fairly resistant to heat. However, hyperthermia influence membrane fluidity, which

in turn could affect the ion pump (Kimmelberg and Papahadjopoulos, 1974).

The $\text{Na}^+ - \text{K}^+$ pump is dependent on ATP as driving power.

We have in an earlier paper found evidence that ATP production is suppressed in human mononuclear leukocytes exposed to hyperthermia above 43°C (Jonsson et al, 1984, 1985). This decrease of ATP pools was found to be parallel to a reduced viability as measured by trypan blue exclusion. This phenomenon has also been observed by other authors for various cell types (Francesconi and Mager, 1979, Ohyama and Yamada, 1980, Lunec and Cresswell, 1983). Reduced ATP pools is expected to have great consequences for cellular life. Especially when there is a demand of energy, which is the case after DNA has been damaged, the energy production might be critical. Reduced ATP production is expected to affect energy-dependent transport mechanisms, which in turn might limit uptake of DNA repair precursor substances and disturb ion pumping. However, hyperthermic reduction of ATP is probably not occurring in all cell lines. Yi (1979) found no change of intracellular ATP pools in the mouse mastocytoma cells exposed to hyperthermia. Similarly, Henle et al (1984) found no significant reduction of cellular ATP in HeLa cells exposed to heat. Also Gerweck et al (1984) found no immediate decrease in ATP following moderate heating under normal conditions.

There are many possible targets for hyperthermia, e.g. chromosomal damage (Dewey et al, 1978), inhibition of cellular respiration and metabolism (Cavaliere et al, 1967, Mondovi et al, 1969, Muckle and Dickson, 1971, Dickson and Shah, 1972). The cell membrane is certainly also a crucial target for heat damage, where lipid composition, fluidity and permeability of molecules and ions may be affected (Yatvin, 1977, Wallach, 1978, Cress et al, 1978, Kwock et al, 1978, Yi, 1979, Yi et al, 1983a, 1983b). The hyperthermia effects on the plasma membrane could result in loss of integrity and ^{cause,} diffusion of

ions along their concentration gradients which then could be one explanation for the observed effects on cation content.

One other possible mechanism for the hyperthermia effect on cation content was considered by Yi et al (1983b). They found that the total intracellular pool of amino acids increased significantly after hyperthermia treatment of mastocytoma cells. The increased pool of amino acids was suggested to act as a source of osmolarity maintaining the cellular volume almost intact. The efflux of K following hyperthermia could then be a cellular response to the increased osmolarity.

The changes in intracellular ion content can alter not only the intracellular pH, critical for survival, but also the activity of DNA repair enzymes (Spiro et al, 1982, 1983) and probably cellular proliferation capacity (Quastel and Kaplan, 1970, Segel et al, 1975).

The present study supports the idea that the plasma membrane plays a significant role in the response of cultured cells to hyperthermia.

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TABLE CAPTION

Table I. Ion concentrations in human lymphocytes as measured by the PIXE method and by atomic absorption. Data obtained from the same blood donor (n=4).

TABLE I

Ion	PIXE method		Atomic abs. spectrometer	
	Content of ion (fmol/cell)	Detection limit (fmol/cell)	Content of ion (fmol/cell)	Detection limit (fmol/cell)
Na	N.D.*	-	3^{+1}	1 ?
Mg	N.D.	-	$0.8^{+0.1}$	0.2
K	$23.8^{+1.2}$	0.02	10^{+2}	2
Ca	N.D.	-	$1.3^{+0.4}$	0.5
Mn	N.D.	0.012	N.D.	-
Fe	$1.26^{+0.05}$	0.01	N.D.	-
Cu	$0.02^{+0.01}$	0.005	$0.03^{+0.02}$	0.02
Zn	$0.08^{+0.01}$	0.008	$0.05^{+0.02}$	0.02
Br	$0.024^{+0.002}$	0.009	N.D.	-
Rb	$0.021^{+0.004}$	0.009	N.D.	-

*N.D. = Not Determined

FIGURE CAPTIONS

- Fig. 1. Schematic drawing of a PIXE analysing/evaluating system.
- Fig. 2. Typical PIXE-spectrum for human lymphocytes. Cell density 0.5×10^6 cells/20 μ l physiological saline.
- Fig. 3. Intracellular ion levels of K, Fe, and Zn versus cell density on the Kimfol foils. Error bars show the standard deviations.
- Fig. 4. Intracellular level of K versus incubation time at 37°C following exposure of the lymphocytes to 37, 42.5 and 44°C for 45 min. Inserted figure shows the K level versus temperature 5 hrs after heat exposure. Significance levels of deviation from contravalue at 37°C are indicated. The cell density was 1.0×10^6 cells/20 μ l physiological saline.
- Fig. 5. Intracellular level of Zn versus incubation time at 37°C. See figure caption to Fig. 4.
- Fig. 6. Intracellular level of Fe versus incubation time at 37°C. See figure caption to Fig. 4.
- Fig. 7. Intracellular level of Na versus temperature 5 hrs after heat exposure. The analysis was done with atomic absorption spectrometer.
- Fig. 8. The detection limits in ng/cm^2 for consecutive elements with PIXE at a routine analysis of a backing Kimfol foil.

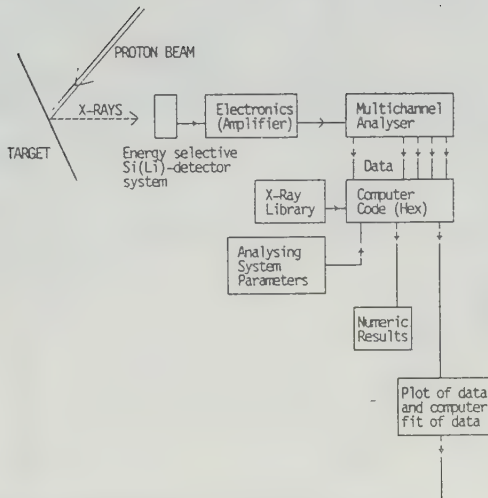


Figure 1

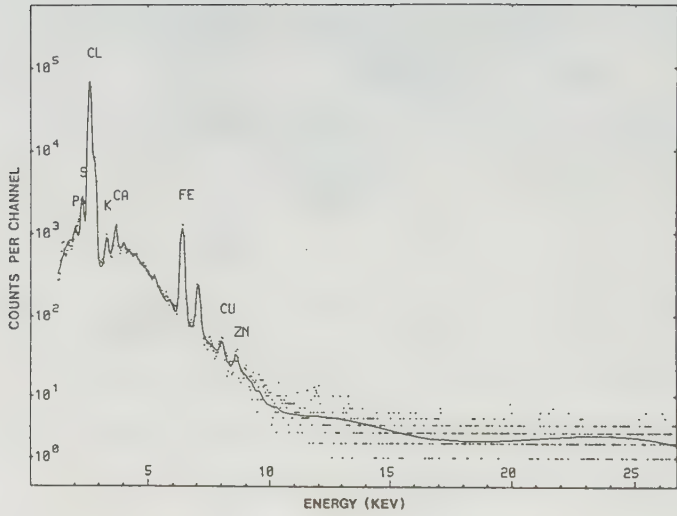


Figure 2

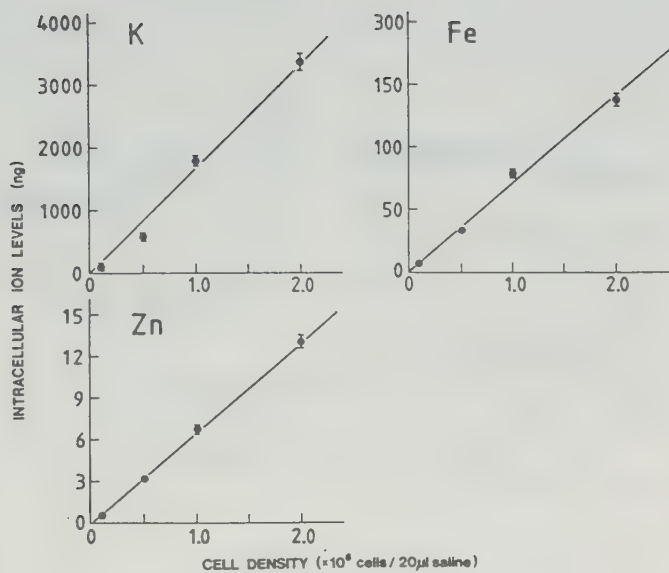


Figure 3

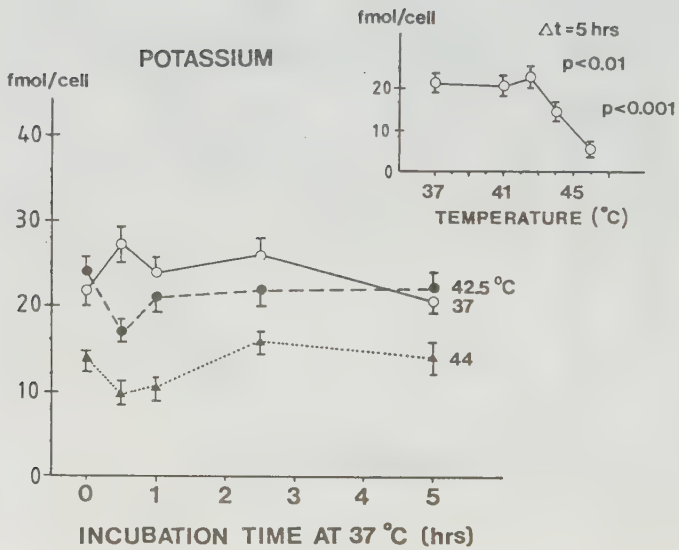


Figure 4

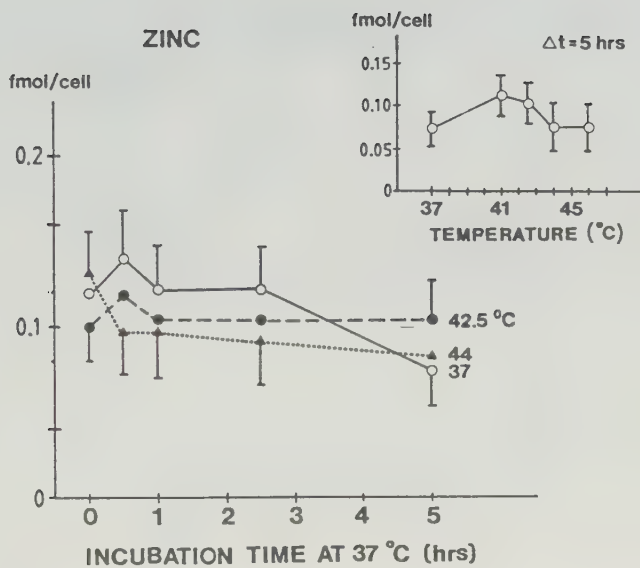


Figure 5

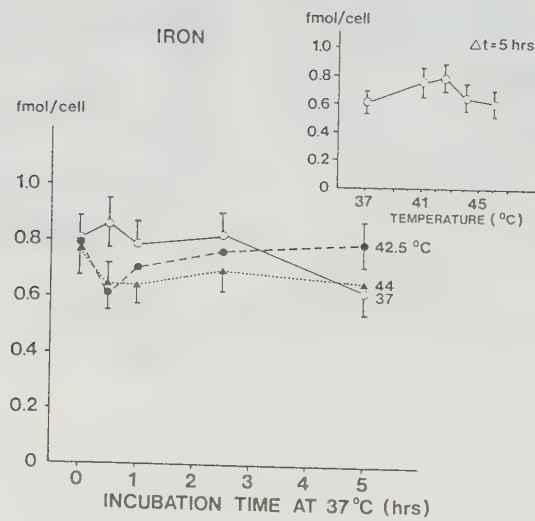


Figure 6

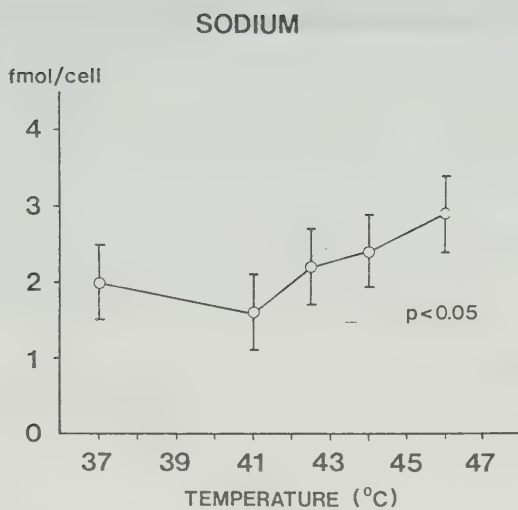


Figure 7

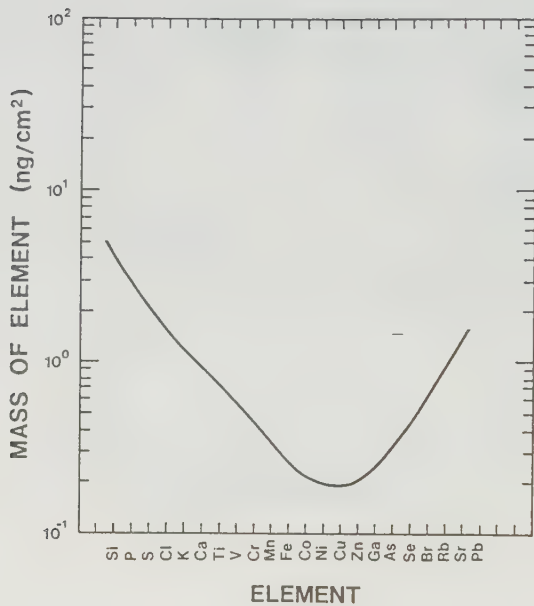


Figure 8

Forward scattering as a tool for measurements of target thickness variation

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Many analytical situations in PIXE require the knowledge of the thickness of the target. Various methods for target thickness determination are discussed, including methods using energy loss measurements. A method consisting of a combination of energy loss determination and forward scattering in a thin carbon foil which allows determination of the target thickness variation over the irradiated sample area is proposed and discussed. The method is demonstrated on a special target with a step-wise increasing thickness. Sources of error and their importance in the final corrections are discussed.

0. Introduction

A common rule of thumb when performing quantitative PIXE (Particle Induced X-ray) analysis is to keep the analysed target either thin or thick. To express this more precisely, the areal mass density should be sufficient small not to change the particle energy during its passage through the target and to allow the corresponding X-ray production cross section to be regarded as constant throughout the target. Also this means that the X-ray absorption in the target should be small or negligible. Thus no corrections have to be applied to the thin-target results. A thick target, on the other hand, stops the particle beam completely. Knowing the major elemental composition of the target, corrections due to the changing X-ray production cross section and X-ray absorption can be applied [1]. For protons with an initial energy of 2.6 MeV, the range in a target of biological matter is below 15 mg/cm². In this case the limit for a sample to be regarded as thin is 1-2 mg/cm², depending on the matrix composition and the lightest element to be analysed. The thick-target limit will be 12-15 mg/cm². The region in between, the intermediate thickness region is often avoided.

There are, however, several situations where PIXE samples not will fulfil the above criteria. A common method of preparing samples of biological origin is to spot a solution onto a thin foil and then allow it to dry. This procedure is used for samples made from human body liquids, such as blood (whole, serum, plasma or protein fractions), urine (directly or in a pre-treated form), sweat etc. The organic matrix of a biological sample is often broken down by dry ashing, the ash residue is usually dissolved in an acid and then spotted onto a foil. After drying, the targets will sometimes have structures which can not be regarded as thin; there may also be crystals or thick rings on the foil. If these targets are regarded as thin, this will lead to an underestimation of the elemental content.

In the same manner, samples from many biological structures that are analysed directly with PIXE have an intermediate thickness. Examples of such samples are sections produced by cutting tissues with a microtome. In microbeam analysis there may be local thickness variations in the sample which will change the number of characteristic X-rays from that part of the sample. If the thickness variation is unknown and the change is not related to the mass variation, this will be regarded as a concentration variation [2].

Aerosol samples are produced by the collection of particles on a substrate which, under certain conditions, can result in a sample which is partly of an intermediate thickness. There can also be individual "coarse particles" each representing a thick target.

To achieve optimal detection limits, it is sometimes useful to use targets of intermediate thickness. However, for such thicknesses, the PIXE results will show too low values. To be able to perform quantitative analysis, the target thickness and composition must be known. Bearing this in mind, and the factors mentioned above, it is clear that there is a demand for a method of measuring the target thickness, and, if possible, also the thickness variation of an inhomogeneous sample within the irradiated area. Below follows a discussion of different methods of determining the target thickness, and some alternative quantification procedures.

1. Methods of determining target thickness simultaneously with PIXE analysis

There are various methods available which can be used to determine the thickness of a PIXE sample.

1.1 Secondary electron Bremsstrahlung (SEB) continuum

In X-ray spectra from PIXE analysis the SEB intensity is a function of the sample mass and composition. For thin samples and with biological matrices this background can be approximated by a linear relation to the mass [3]. Some drawbacks of the method are that with "less thin" targets self absorption of X-rays will occur which must be compensated for. Second, there are also contributions to the background from high- and low-energy tails. Third, discharges from a charged sample will cause emissions of photons, which drastically can increase the background. To obtain an accurate mass determination, adequate pulse statistics is required. Hence a relatively large part of the background has to be used, but this increases the risk of including peak tails in the background.

1.2 Absorption of X-rays

By using the absorption of X-rays from one internal standard (given by the ratio of the amount measured by PIXE to the known amount) it is possible to calculate the target thickness. This requires that the matrix is known and that any difference in the ratio of measured to expected amount is completely due to absorption. The standard has to be very carefully mixed into the sample to meet the above demand. Using a standard for absorption measurements also implies that it can not be used at the same time as a current monitor in PIXE analysis [4], or as an element for normalization.

Another way of performing absorption measurements is to dope the sample with two standards emitting X-rays with as different absorption coefficient in the matrix as possible, and then to measure the ratio between them [5]. However, practical reasons limit the choice of standards. The lightest suitable element when working with biological matter would be titanium or vanadium, as lighter elements can be expected to occur naturally in the sample. A typical choice for the heavy element is yttrium or silver.

To make possible an accurate measurement, there should be as large a ratio as possible between the thick-target correction factors for the two elements. From fig. 2 (see below section 2) it follows that a suitable combination would be $Z \approx 23$ and $Z \approx 56$. However, with increasing atomic number, the X-ray production cross section decreases, and requires a higher doping concentration of this element. Also, the heavier element of the dopants must be chosen in such a way that its L X-rays do not interfere with K X-rays from elements of interest.

There are some other limitations when using ratios of two internal standards. To be able to determine the thickness with an error of less than 0.1 mg/cm^2 , it is necessary to have extremely good statistics in the peaks from the standards. Let A_A and A_B be the amounts of the two standards and N_A and N_B their peak areas. If Y_A and Y_B are the thin target yields and f_A and f_B the thick-target correction factors it

follows that:

$$A_A/A_B = (Y_A f_A N_A)/(Y_B f_B N_B)$$

Let R be the ratios of the thick-target correction factors. From fig. 1 it can be seen that R changes from 1.0 to 0.90 when the target thickness changes from 0.0 to 10.0 mg/cm². Thus

$$R = f_A/f_B = A_A/A_B \cdot (N_B Y_B)/(N_A Y_A)$$

Assuming Poisson statistics, the relative standard deviation in R can be written

$$(\sigma_R/R)^2 = (\sigma_{N_B}/N_B)^2 + (\sigma_{N_A}/N_A)^2 \approx 2 (\sigma_{N_A}/N_A)^2$$

As $R \approx 1$ and assuming gaussian peaks, it follows that

$$(\sigma_R)^2 \approx 2/N_A$$

Allowing an error in the thickness determination of 0.1 mg/cm², it follows from fig. 1 that $\sigma_R \approx 0.001$ and thus

$$(2/N_A)^{0.5} \approx 0.001$$

$$\Rightarrow N_A \approx 2 \cdot 10^6$$

This requires long irradiation times and/or high doping concentrations.

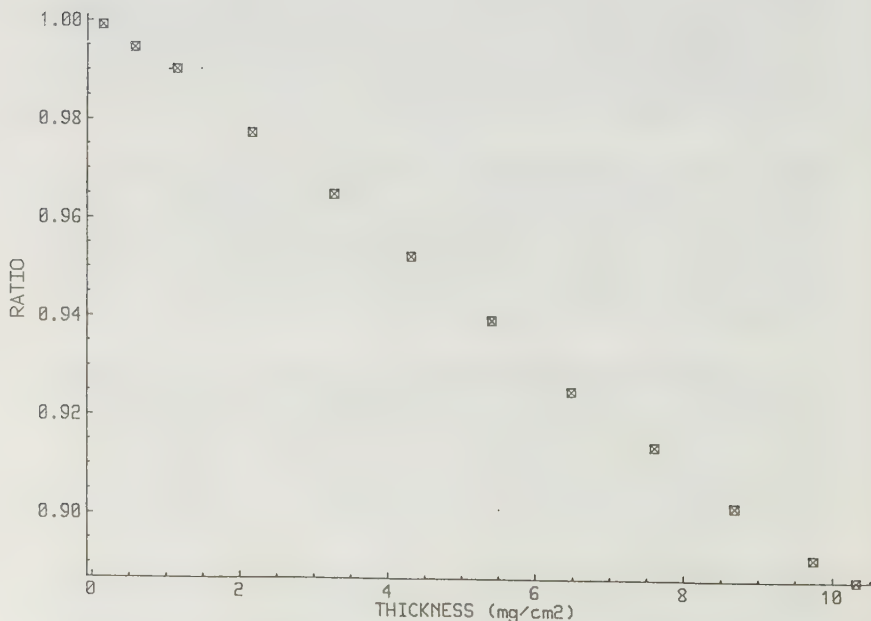


Fig. 1 Ratio of the thick-target correction factors for vanadium and strontium for different target thicknesses for a biological matrix.

1.3 Backscattered particles

The number of elastically backscattered particles can be used to measure the target thickness [2]. The number of counts in the detector is proportional as follows:

$$N \sim \sum_i C_i \int_{E_0}^{E_1} \sigma_i(E) dE t$$

C_i is the concentration fraction of the matrix element i and $\sigma_i(E)$ the energy-dependent scattering cross section for element i . The integration is carried out from the energy of the incoming particles, E_0 , to the exit energy, E_1 , resulting from their passage through the specimen of thickness t . If the scattering cross section is constant within the energy interval of the particle path, the summation can easily be performed. For a proton beam in the energy range 2.0–2.55 MeV, the scattering cross sections for C, N and O are essentially constant, apart from a resonant structure for N in the region of 2.3 and 2.5 MeV. The upper limit on the proton energy that can be used is set by the existence of resonances at higher energies, and the optimization of the general analytical conditions for PIXE analysis. With a proton energy of 2.55 MeV, the upper limit on the sample thickness will be about 4 mg/cm² [6].

1.4 Energy loss of particles

The energy loss of particles passing through the target is a function of the particle charge and velocity and the target thickness and composition. There are several ways of determining the particle energy after the target.

i) In the case of protons, the following inelastic nuclear scattering reaction in aluminum: $^{27}\text{Al}(p, p'\gamma)^{27}\text{Al}$ with $E_\gamma = 1013$ keV, can be used. The γ -yield versus proton energy is a steep linear function between 2.75 and 3.0 MeV, but not at lower energies. By dumping the beam in an aluminum Faraday cup, and measuring the γ -ray intensity, the target thickness can be calculated [7,8]. To make use of the linear part of the yield curve, the initial proton energy must be 3.0 MeV. An energy loss of 0.25 MeV corresponds to a target thickness of about 2 mg/cm². To be able to analyse thicker targets, or to use a lower initial proton energy, knowledge of the exact behaviour of the Al γ -ray yield curve at the corresponding proton energy is required.

ii) Forward scattering of particles from the target may also be used. A detector is placed at a forward angle and the energy of a characteristic peak in the scattering spectrum is used to determine the energy loss and hence the thickness of the target can be calculated. The energy of the particles after scattering depends both on which element it was scattered off, and the energy loss suffered during passage through the sample. This method will work only for rather thin targets [9]. For thicker targets, there will be an unresolvable mixture of peaks from different elements which will render good identification and the following energy loss determination impossible.

iii) Backscattering of particles from a thin foil placed after the target is another possibility. The energy of the protons can be directly determined in the spectrum of the scattered particles. To

obtain a useful peak it is important to have a very thin foil. By using a heavy element deposited as a thin layer on a plastic foil, the difference in kinematic factors (see below) ensures that the peak from the heavy element is well separated from the peaks from the plastic foil. In the case of Au deposited on a plastic foil, the kinematic factors for proton scattering in an angle of 160° will be 0.78 for oxygen and 0.98 for gold, which is a sufficient separation.

iv) Forward scattering of particles in a thin foil placed after the target [9,10] can be used. As in case (iii) above, the proton energy can be directly taken from the peak position in the spectrum. The scattering foil must be mono-elemental as in forward-angle scattering the small difference in kinematic factors for different elements will not give a sufficient separation of the peaks for light and heavy elements. The kinematic factors for protons scattered from oxygen and gold at 45° are 0.964 and 0.997, respectively. Compared with method (iii) above the yield for forward scattering is much higher, which enables the use of a tighter collimation and reduces the time needed to obtain good statistics in the spectrum. Forward scattering is the method proposed and discussed in this paper.

2. Alternative ways to perform quantification of samples of intermediate thickness

There are other solutions to the problem of correcting the PIXE results for the effects of a sample of intermediate thickness, which can be used in some special cases, however, with a varying degree of accuracy. These are a) to normalize to an internal standard, b) to do absorption measurements or c) other methods for estimation of the thickness.

In choosing between these alternatives the major question is what error you can expect in using the methods a) to c) compared with more stringent correction methods. Neglecting the thickness will give errors equal to the correction factors applied in intermediate thickness corrections. These correction factors are shown in fig. 2 for a biological matrix. The factors have been calculated with the code THICK-500 (see Appendix) for a typical geometry used during analysis, i.e. the angle between the X-ray detector and beam is 135° , and that between the target normal and the beam is 157.5° . It can be seen that the correction curves vary smoothly with Z.

Correction curves are also given for a non-biological matrix with heavier elements, (here exemplified by the matrix: C, Al, Si, P, S, K and Ca 10% each, O 10%), see fig. 3. Such a matrix could result after ashing of a biological sample.

The target thickness dependence in PIXE analysis can also be expressed as the thickness of the surface layer from which 10, 50 and 90% of the characteristic X-rays that reach the target surface are emitted (see figs 4 and 5).

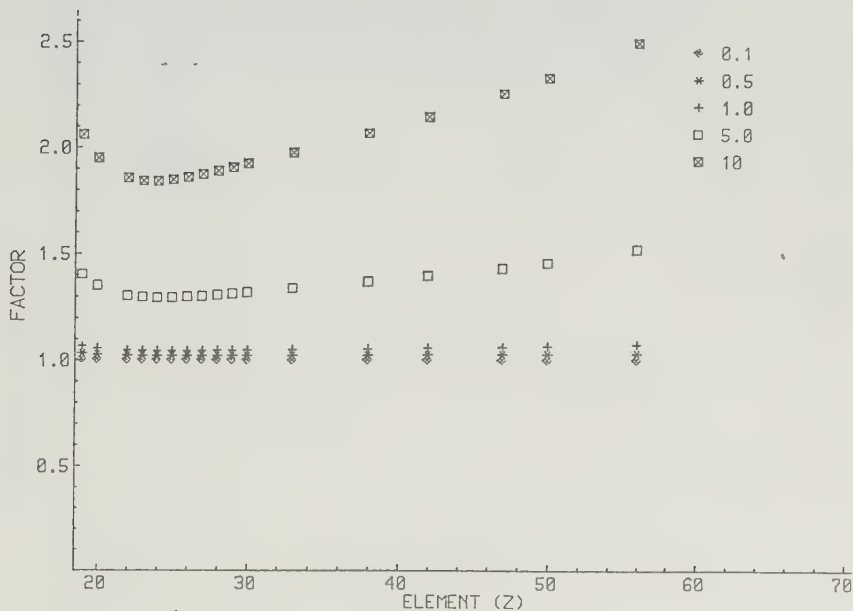
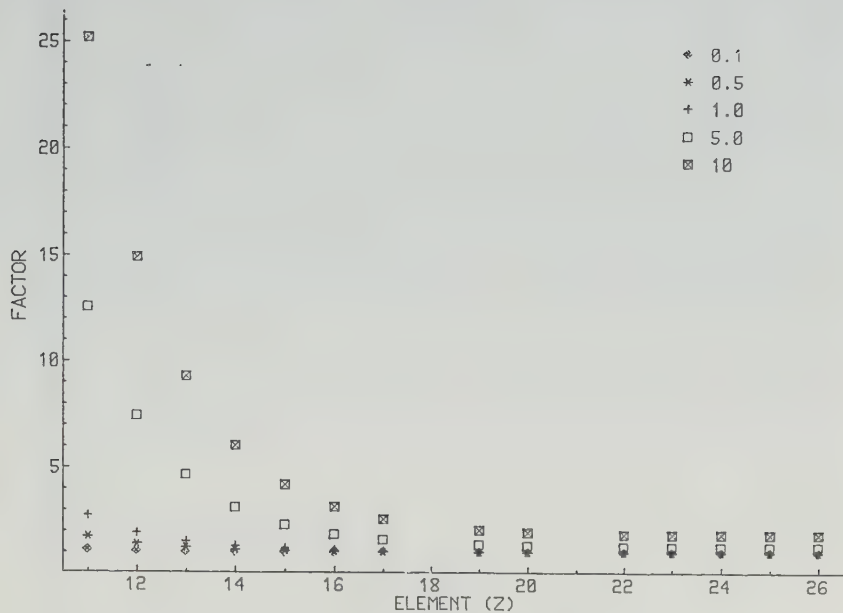


Fig. 2a,b Thick-target correction factors as a function of Z (a, $Z=11-26$; b, $Z=20-56$) for a Kapton[™] matrix (simulating a biological matrix) with target thicknesses of 0.1, 0.5, 1.0, 5.0 and 10.0 mg/cm^2 . Protons with an energy of 2.6 MeV have been assumed. The angle between the X-ray detector and beam is 135° , and that between the target normal and the beam is 157.5° . *note the change in the scale on the axes.

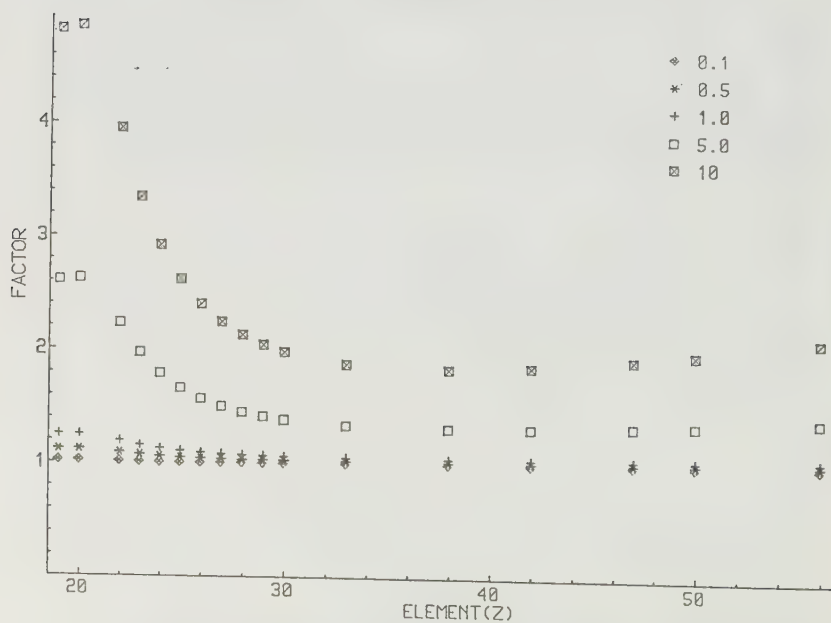
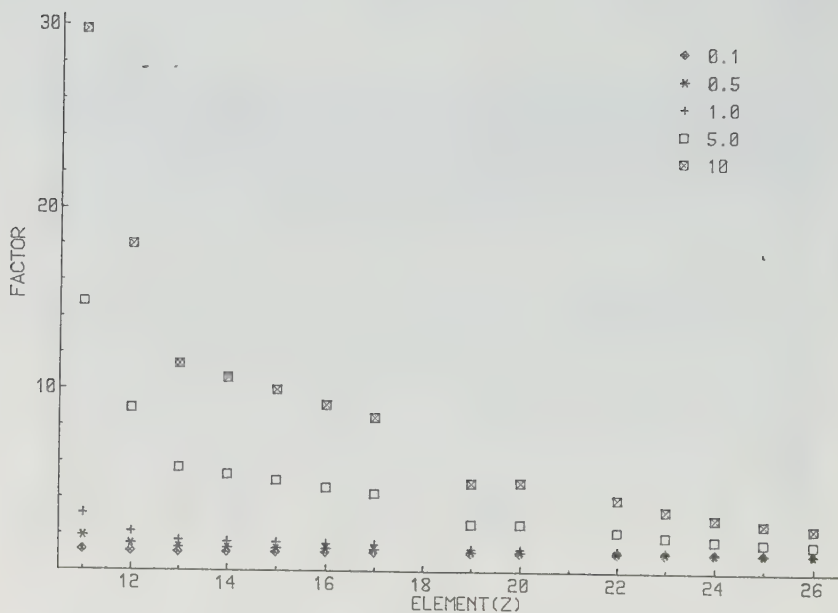


Fig. 3a,b Thick-target correction factors as a function of Z (a, Z=11-26; b, Z=20-56) for a non-biological matrix with target thicknesses of 0.1, 0.5, 1.0, 5.0 and 10.0 mg/cm². Matrix: C, Al, Si, P, S, K and Ca 10% each, O 30%. The sample geometry and the proton energy are the same as in fig. 2. Note the change in the scale on the axes.

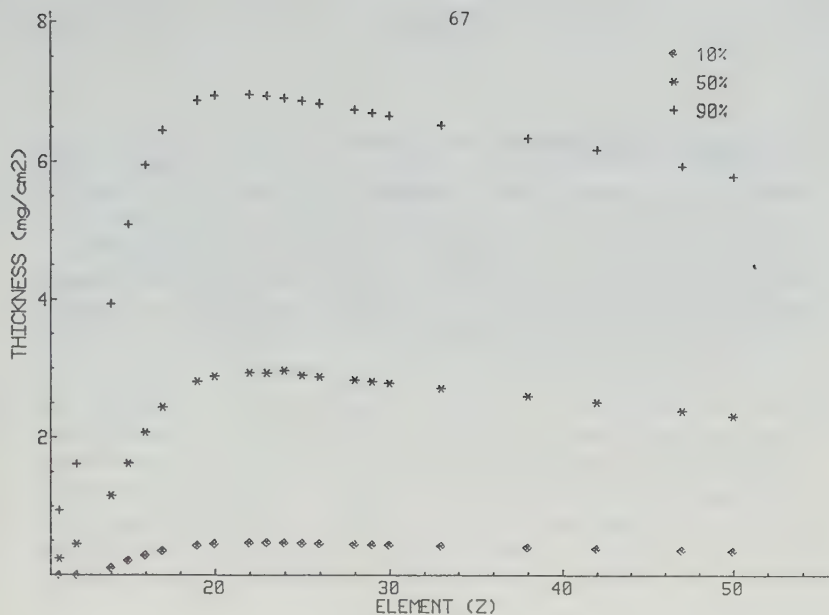


Fig. 4 Effective PIXE depth for a thick biological matrix (H 5%, C 76% and O 19%). The curves show at from which thickness of surface layer 10, 50 and 90 % of the emitted X-rays are received as a function of atomic number Z . The sample geometry is the same as in fig. 2.

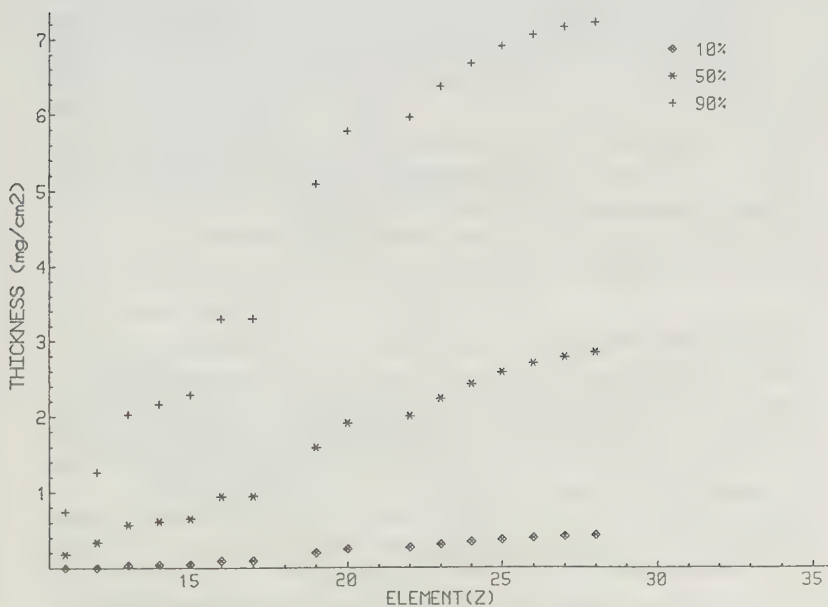


Fig. 5 As fig. 4 but with the matrix H 5% and N 5%, C 20%, O 40 % and Al, Si and S 10 % each.

2.1 Internal standard

Normalizing to added internal standards (e.g. vanadium, yttrium) is equivalent to defining the correction curve to be 1.0 for the standard. The errors for other elements will then equal the ratio between the correction factor for this element and the actual correction factor of the standard. However, for the lightest elements the deviations can be large. The calculated errors for a biological matrix with a thickness of 5 mg/cm^2 are shown in fig. 6.

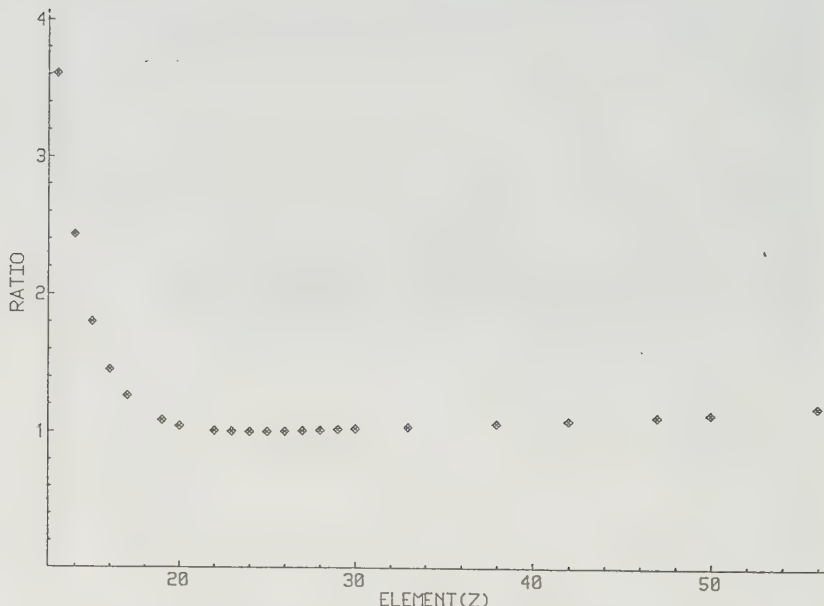


Fig. 6 Ratio of thick-target correction factors to that of the internal standard (vanadium), for different elements when normalizing to the internal standard for a biological matrix, sample thickness 5 mg/cm^2 . The sample geometry is the same as in fig. 2, and $E_p = 2.6 \text{ MeV}$

2.2 Absorption measurements

A method is described by Carlsson et al. [11] that makes use of a beam of collimated X-rays passing through different parts of the target. The emerging X-rays are measured in a Si(Li)-detector, thus enabling the target thickness distribution to be determined. When using uniform polymer foils of known compositions, and with thicknesses ranging from $1.5 - 11 \text{ mg/cm}^2$, the inaccuracy of the correction procedure is stated to be better than 5%. A major drawback with the method is that it is time consuming and can not be performed at the same time as the PIXE analysis.

Another method of determining the mass is to use attenuation of β -particles. In a special set-up that has been developed and described by Hedberg [12], the mass of aerosols collected on Nuclepore filters is measured. This set-up has been specially designed to measure a

small amount of a sample (100-500 $\mu\text{g}/\text{cm}^2$) deposited on a thicker filter (1 mg/cm^2).

2.3 Other methods of thickness determination

Weighing the sample and measuring its area (after the analysis), could be used as a method of estimating the thickness if the sample is homogeneous and has a constant thickness. If this method is combined with normalization to an internal standard, the final results after correction will be improved. A major drawback with this method is that the estimation procedure can not be performed simultaneously with the PIXE analysis, but must be made at another time. This involves the risk that the target has meanwhile changed its properties, leading to an erroneous thickness estimation. The sample may, for example, be hygroscopic and increase its weight by absorbing water from the air. Another limitation to this procedure is that if the target thickness is not constant across the sample, the correction applies only to the mean thickness of the sample. Thus there may be an error in the correction, see also section 5.2.2 below.

3. Energy-loss and forward scattering measurements

When charged particles pass through a target several processes occur; they lose energy, their energy spread is increased and they are scattered; all these processes are of a statistical nature. In the discussion below the initial energy is of importance. One can always argue about the choice of energy. To be able to determine the thickness of thicker targets, the particle energy chosen should be high, as the stopping power decreases with increasing energy. This would, at the same time, lower the energy deposited in the target and hence the heat production. However, to minimize the effect of nuclear resonances in the energy interval of interest, the energy should be low. The main argument for using a certain energy is that it optimizes the overall analytical PIXE sensitivity [13]. In this study, protons with an initial energy of 2.6 MeV were used.

3.1 Theory

3.1.1 Energy loss and energy straggling

The process of energy loss is statistical and the distribution of particle energies after passing through a target is approximately gaussian. A thicker target will yield a broader distribution of particle energies, a phenomenon called energy straggling. A model of the corresponding standard deviation is initially given by Bohr [14] and states that

$$\Omega = 8.85 Z_1 \cdot (2Z_2/A_2 \cdot \Delta\xi)^{0.5} \quad [(\text{keV})]$$

where Z_1 is the atomic number of the projectile, Z_2 and A_2 are the atomic and mass numbers of the target, respectively, and $\Delta\xi$ is the areal mass density in mg/cm^2 .

The energy loss, ΔE , depends on the initial particle energy, the areal mass density and the elemental composition of the target. The energy

loss can be calculated if the stopping power of the matrix is known. This can be done using different approximative formulae. In this paper the energy loss was calculated with the computer code THICK-500 which is used for thick-target corrections to PIXE results, see appendix.

As an illustration, the energy loss of protons, with an initial energy of 2.6 MeV, as a function of target thickness is given in fig. 7 for some different matrices. One can note the rapid energy loss in the H matrix compared with other elements.

3.1.2 Theory of scattering

For a detailed discussion of the theory of scattering, see, for example, Chu [15]. Some important concepts are mentioned here.

3.1.3 Kinematic factor

The energy change upon scattering through an angle θ is given by

$$\text{the kinematic factor } K_m = \left[\frac{M_1 \cos \theta + (M_2^2 - M_1^2 \sin^2 \theta)^{0.5}}{M_2 + M_1} \right]^2$$

where M_1 and M_2 are projectile and target mass respectively, see fig. 8.

3.1.4 Elastic scattering cross section

The elastic scattering differential cross section $d\sigma/d\Omega$ is given by

$$d\sigma/d\Omega = \left(\frac{Z_1 Z_2 e^2}{4 E_0} \right)^2 \frac{4}{\sin^4 \theta} \frac{\{ [1 - ((M_1/M_2) \sin \theta)^2]^{0.5} + \cos \theta \}^2}{[1 - ((M_1/M_2) \sin \theta)^2]^{0.5}}$$

where

Z_1 and M_1 are atomic number and mass of the projectile,

Z_2 and M_2 are the corresponding number and mass for the target atom,

E_0 is the initial energy (in MeV) and e^2 equals $1.4398 \cdot 10^{-13}$ [MeV cm]

3.1.5 Resonant behaviour

For certain projectile energies nuclear reactions occur in the target. These reactions involve rearrangements in the nucleus followed by an emission of radiation and/or particles. An energy scan of the scattering yield will, under such circumstances, give a resonant peak in the resonance region. The shape and amplitude of this peak is highly dependent on the scattering/observation angle. When using protons with an initial energy of a few MeV, it is important to bear in mind the nuclear elastic scattering reaction $^{12}\text{C}(p,p)^{12}\text{C}$ at $E = 1.698$ MeV. The consequences of this will be discussed below.

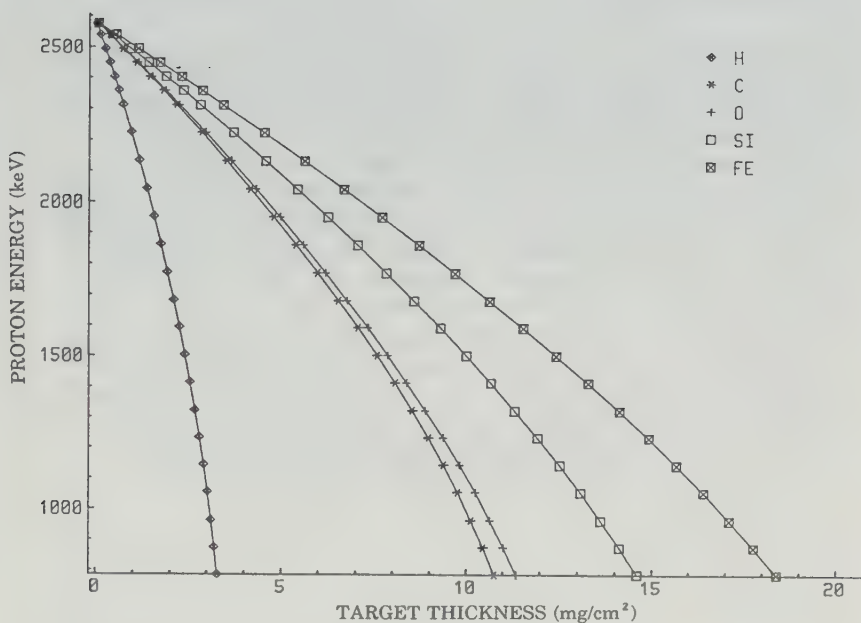


Fig. 7 The proton energy (initial energy of 2.6 MeV) as a function of the target depth for different elements. Note the rapid energy loss when using H as matrix compared to other elements. The target normal is rotated 22.5° relative to the beam.

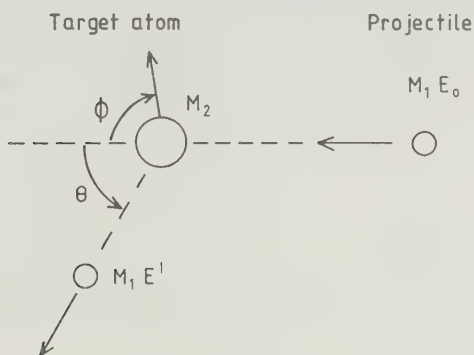


Fig. 8 Scattering angle geometry.

3.2 Experimental details

As stated above, protons with an initial energy of 2.6 Mev, produced by the 3 MV Pelletron tandem accelerator in Lund, were used in the experiments. The energy loss in the sample was measured by forward scattering of the protons in a carbon foil placed 5 cm after the sample. The scattered protons were detected in a surface-barrier detector, see fig. 9 for set-up. To prevent stray protons from entering the carbon foil and becoming scattered, a pair of collimators (the first with a smaller hole) was placed in front of the scattering foil. The surface-barrier detector was placed 7 cm from the scattering foil at an angle of 45 degrees. To achieve good collimation, two collimators were placed between the detector and the foil. The beam size was either 1 or 3 mm depending on sample size.

To investigate the variation in the scattering cross section in carbon with energy, the scattering yield from the thin carbon foil was measured at 45 degrees for proton energies between 0.90 and 2.50 MeV. The yield curve is shown in fig. 10. It can be seen that the yield increases for decreasing energies, this is due to the Rutherford elastic scattering cross section (RESCS) which is proportional to $1/E_0^2$. Dividing by the RESCS gives the spectrum shown in fig. 11, i.e. a more constant yield apart from the resonant peak.

When having an inhomogeneous sample with a thickness greater than 5-6 mg/cm², the protons are slowed down to 1.7 MeV and below. The corresponding scattered proton spectrum shows a peak originating from this resonance, fig. 12. In evaluating such a spectrum it is necessary to correct for the variation in both RESCS and the resonant behaviour, see section 3.3.2 below.

3.3 Spectrum evaluation

One part of this work includes the development of a method that allows the determination of the thickness variation within the irradiated area of the sample. The method proposed here is based on the hypothesis that the particle spectrum itself contains information about the thickness variation in the sample, and that it can be deconvoluted to reveal this thickness variation. It is assumed that the spectrum is a superposition of a finite number of "energy elements", each representing a specific energy loss, which in turn corresponds to a certain thickness traversed by some part of the beam. By unfolding the spectrum, by reconstructing these "energy elements", the energy distribution corresponding to the situation just after the target can be obtained. When combined with the proton energy loss function for the matrix irradiated, the thickness distribution of the sample can be calculated. This final distribution gives a simplified picture of the sample thickness distribution, and is used when performing thick-target corrections. The thick-target corrections will be improved compared with those based on the assumption of only one thickness of the sample.

To understand the shape of the particle spectrum it is necessary to discuss the processes that are involved at its creation, and which approximations have to be performed in deconvolution the spectrum.

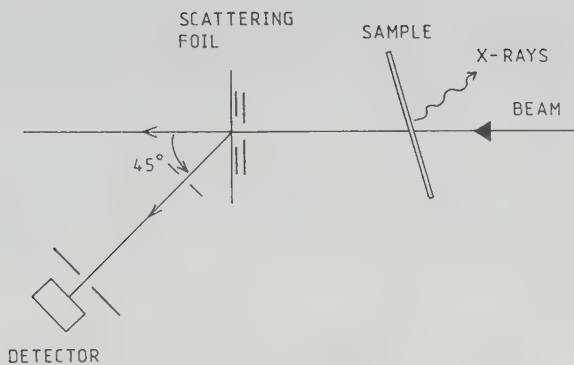


Fig. 9 Schematic diagram of the experimental set-up.

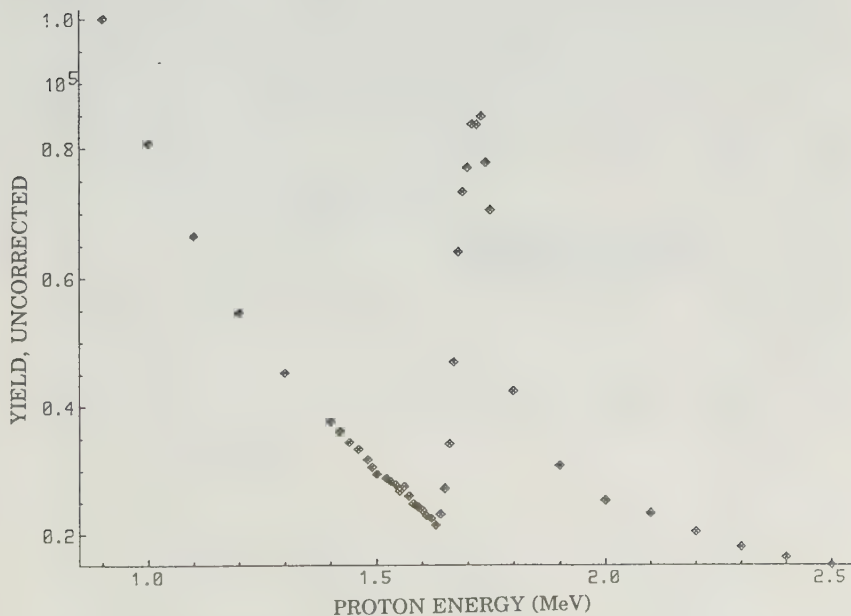


Fig. 10 Measured scattering yield versus incident proton energy at $\theta_{\text{lab.}} = 45^\circ$ for $^{12}\text{C}(\text{p,p})^{12}\text{C}$.

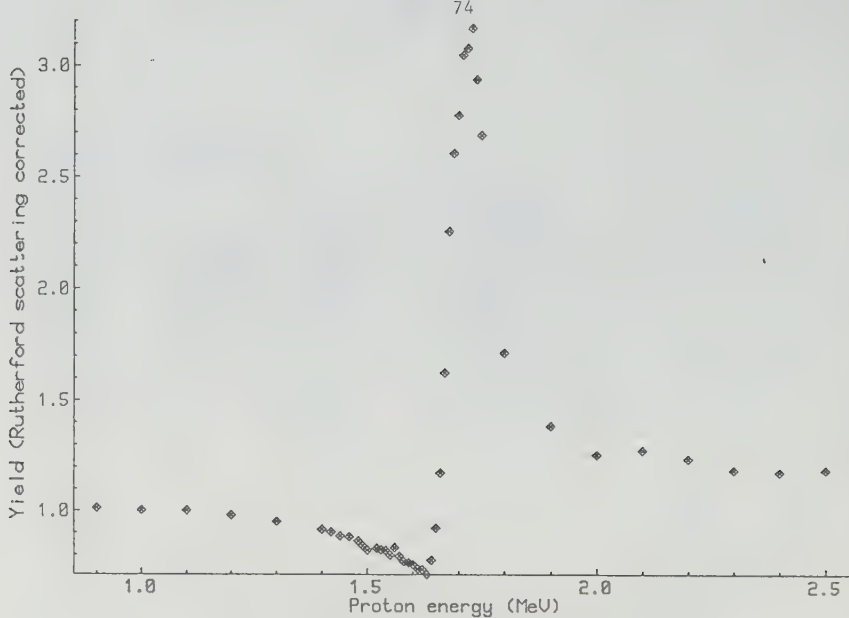


Fig. 11 The yield curve of fig. 10 divided by the Rutherford elastic scattering cross section, and normalized at $E = 1.0$ MeV.

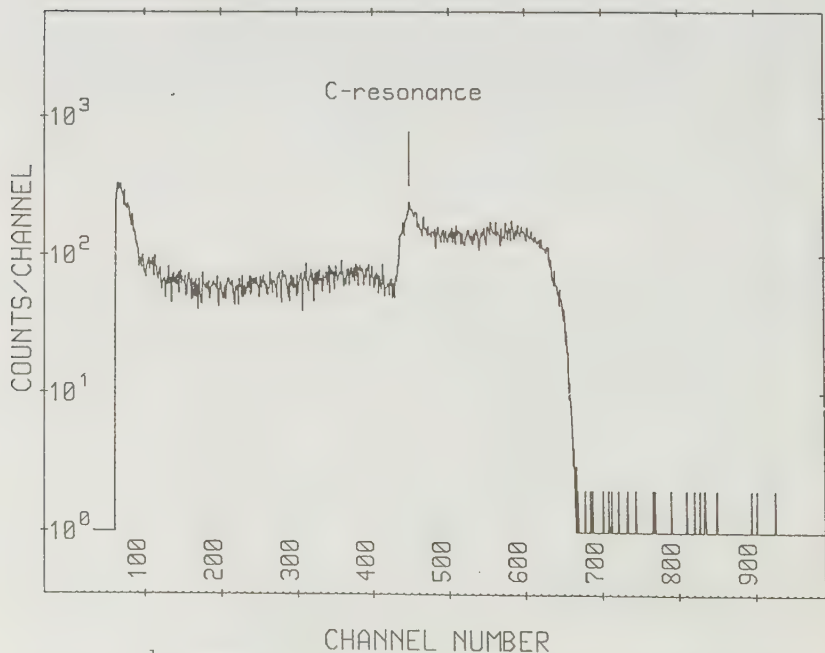


Fig. 12 A ^{pulse} height spectrum from a thick inhomogeneous biological sample. The initial proton energy was 2.6 MeV, the beam scattered in a carbon foil placed after the target and detected at an angle of 45° . Since the protons are slowed down to 1.7 MeV, there is a peak from the carbon resonance.

3.3.1 Discussion of physical mechanisms involved in the transformation from target thickness distribution to pulse height spectrum

In the worst case the irradiated sample will have a heterogeneous elemental composition and an irregular thickness distribution. The approximations made here are that the main elemental matrix composition is constant throughout the sample, and that the irregular thickness can be regarded as a composition of a finite number of discrete thicknesses.

As the beam passes through the sample the protons are slowed down by an amount depending on the thickness of the sample and its composition. The width of the energy distribution will be a function of the target thickness due to the energy straggling. Since the target is of variable thickness, the protons will be slowed down differently in different parts of the beam. Thus, after passing through the sample, the energy distribution of the beam will be transformed from an initially very sharp distribution (given by the accelerator system), to a broader one being a function of the sample thickness. The approximation made here is that, except for proton slowing-down and energy straggling, no other phenomenon, e.g. multiple scattering or nuclear reactions, will affect the energy distribution (see also section 3.3.3 below).

The energy distribution of the protons entering the carbon foil is a simple function of the target thickness distribution. After scattering in the foil the proton beam will be transformed in different ways: a) the overall energy distribution will be changed according to the kinematic factor giving $E' = E_0 \cdot K_m$, i.e. a linear energy transformation, b) the cross section for elastic scattering varies as $1/E_0^2$, leading to an energy-dependent scaling, c) at proton energies around 1.7 MeV the resonance in the scattering cross section for carbon results in rapid change in the yield, d) non-central particles will be scattered at other angles than the central ones and reach the detector with different values of the kinematic factor, which will give a broadening effect, see 5.1.

The energy resolution in the detector is limited, but the resulting broadening is small compared with the energy straggling in the sample, and can be disregarded. Low-energy tailing can partly originate in the detector, other sources may be multiple scattering in the target and slit scattering.

In the acquisition system, amplifiers, ADC's, effects of dead time and pulse pile-up etc. can influence the transformation from energy distribution to pulse height distribution. To minimize pile-up, the count rate must be limited.

3.3.2 Deconvolution of the spectrum

Let 'a' (cm^2) be the total area of the sample and a_i a part of it having a thickness ξ_i (mg/cm^2). Protons that pass through a mass element with the area a_i in the sample, are assumed to take a gaussian shaped energy distribution. This is characterized by the centroid energy (which equals the mean proton energy after passage of the mass element), the FWHM given by the straggling (which varies as the square root of the areal mass density ξ) and the intensity, which

is proportional to the area a_i of a mass element. Contributions from mass elements of equal thicknesses, but with different areas ($\xi_i = \xi_j$, $a_i \neq a_j$) will fall in the same energy range. Thus the total intensity in an energy interval is proportional to the total area of the corresponding mass elements ($\sum a_i$, $\xi_i = \text{constant}$). Mass elements of nearly equal thicknesses give rise to partly overlapping energy distributions.

Below follows a description of the procedures performed on the pulse height spectrum to transform it back into a target thickness distribution.

First, neglect the effects of ADC's or detectors on the spectrum, i.e. assume that the collected spectrum gives a true image of the energy distribution after the scattering foil. (This assumption should be reasonable if the count rate is low enough to minimize pile-up.)

Second, correct for the RESCS at the scattering foil by multiplying the spectrum by E^2 , see fig. 13a. Tight collimation is required since no corrections are applied for broadening due to non-central protons. If the protons have been slowed down to 1.7 MeV in the target, a peak due to the carbon resonance is present in the spectrum. The yield curve at the carbon resonance is a rapidly changing function of the energy and of the scattering angle. To correct for the resonance means that the spectrum in the resonant region should be divided by factors describing the form of the resonance. From fig. 11 it is seen that there is a change in the yield of about a factor 1.2 when passing the resonance. Data points on the high energy side of the resonance are thus divided by the factor 1.2. No other correction for the resonance is performed in this model, instead the data in the resonant region are replaced by a linear function fitted to the data in the regions close to, but outside the resonance. If there is no change in the slope of the energy distribution at this point, the effect of this approximation is small. However, if there are "structures" in the distribution, they will be lost in this procedure. The corrected pulse height spectrum now corresponds to the energy distribution seen after the target.

The third step is the transformation from energy distribution to target thickness distribution, and it is assumed that: a) the matrix composition of the target is constant and known in the irradiated volume (the allowable deviations from this assumption depend on the matrix), and b) multiple scattering is negligible. In order to simplify the calculations the continuous target thickness distribution is approximated by a histogram, i.e. the target is regarded as a composition of a finite number of mass elements each having a constant thickness. Similarly, the continuous energy distribution after the target is approximated by a histogram.

To convert the corrected pulse height spectrum (which corresponds to the energy distribution after the target) into an energy histogram, the following procedure is performed: First, gaussian peaks with a FWHM corresponding to the straggling at the centroid energy are set up, their distributions are added and fitted to the observed overall

spectral distribution, see fig. 13b. This fit is performed with a minimum* number of gaussian peaks. Second, the areas of the fitted peaks and their peak energies are replaced by a histogram, see fig. 13c. The centre of each column now represents the mean proton energy after passing through mass elements with thickness ξ_i , and the area of the column is proportional to the total area of these mass elements ($\sum a_i (\xi_i = \text{const.})$).

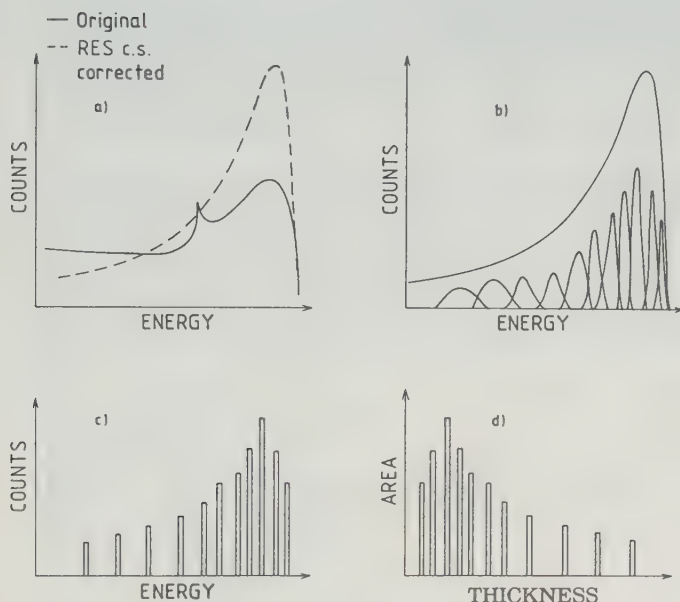


Fig. 13 Schematic representation of the spectrum deconvolution.

a) original and RESCS corrected yields, b) gaussian peaks fitted, c) step representation of peaks and d) final thickness distribution.

* The minimum number of gaussian peaks is the minimum number that produces a smooth curve upon adding the peaks together, taking the varying FWHM into account. If setting up a less number of peaks, their added distribution will fluctuate around the peaks, and increasing the number over the minimum will not yield any extra information.

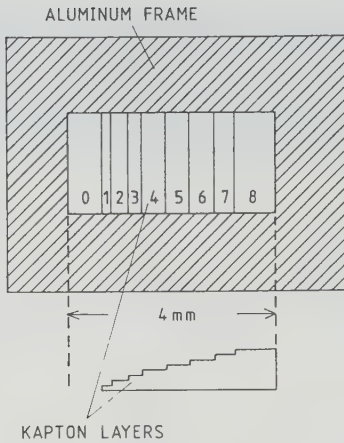


Fig. 14 The Kapton foil multilayer sample. Each sheet of foil is $7.5 \mu\text{m}$ thick (1.4 mg/cm^2). The thickness increases stepwise from 1 to 8 layers. To obtain a well defined target area the sample was masked with aluminum foils forming a rectangular target area.

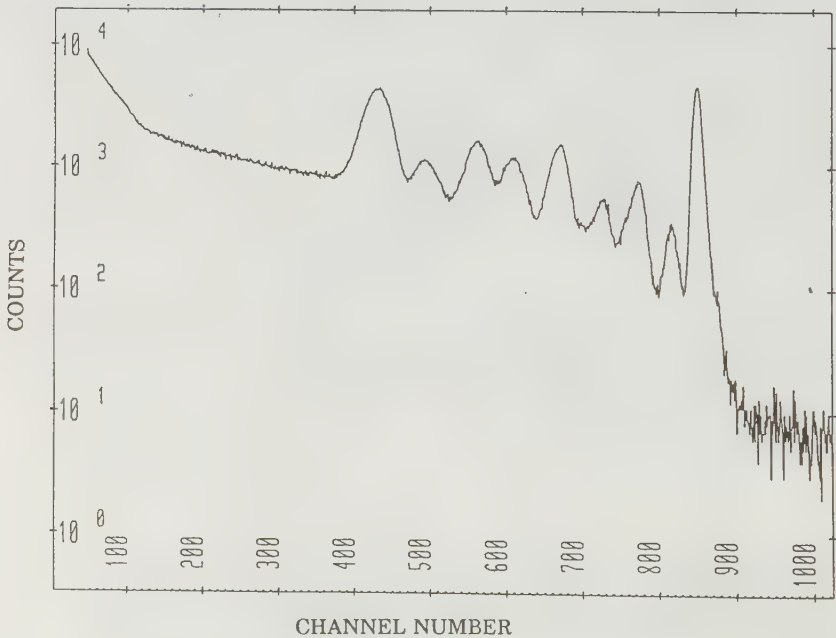


Fig. 15 Spectrum from a Kapton multilayer sample. The peak most to the right corresponds to no layer, going leftward the peaks represents increasing number of layers.

In the final step, the energy histogram is converted into a thickness distribution, see fig. 13d. By calculating a non-linear function, $E=E(m)$ for the given matrix, each energy interval in the histogram can be converted into a column in the thickness histogram, where the area of each column is a measure of the area in the corresponding thickness interval.

3.3.3 The spectrum evaluation model applied to a multi-layer sample

To demonstrate the technique, a special target, $4 \times 2 \text{ mm}^2$, was constructed with step-wise increasing thickness from eight layers of $7.5 \text{ }\mu\text{m}$ thick Kapton[™] foils, see fig. 14. To obtain a well defined irradiation area, the sample was screened with thick aluminum foils thus forming a rectangular target area. The sample was irradiated with a beam larger than the target area, and the particle spectrum shown in fig. 15 was obtained.

The following treatment was then performed: a background was subtracted (see below), the spectrum was multiplied by E^2 to correct for the change in Rutherford elastic scattering cross section, gaussian peaks were fitted to the spectrum and, finally these peaks were replaced by rectangular peaks having the same area as the gaussians. The areas corresponding to energies higher than 1.7 MeV were divided by the factor 1.2, and the peak corresponding to the 6:th layer was omitted from the calculations, as it included the resonance peak, which was not corrected for.

Fig. 16 shows the relative area of each Kapton layer calculated with the method above, and as obtained by measuring in a microscope. The uncertainties, (see also below), for the optically measured points, expressed as standard deviations, were estimated to be $\pm 5\%$, and the corresponding for the points calculated from the spectrum to be $\pm 10\%$. From the data, it is found that 4 points out of 8 overlap when using the 10 % uncertainties, and 7 out of 8 if using a value of 20 %.

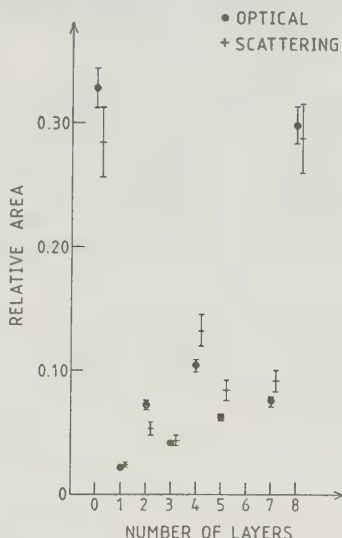


Fig. 16 Comparison between areas of different layers in the Kapton multilayers sample measured by the scattering technique described here and optically in a microscope. The error-bars represent $\pm 5\%$ for the optically measured points and $\pm 10\%$ for the points measured by scattering technique. The data sets are normalised to have the total area equal one.

Some comments on this are: measuring the areas of the layers in microscope is complicated by the fact that the layers are not in the same plane, even a single layer is not perfectly plane. Second, in its irradiation position the sample is rotated an angle of 22.5° . Thus the ratios of the projected areas, as seen by the beam, could differ from the ratios determined in microscope. This yields some extra uncertainty to the optical area determinations.

During normal irradiation, the sample is either smaller than the beam or larger. In this case, however, the sample is limited by an aluminum mask which also limits the beam size. Scattering of protons may occur from the edges of this mask, and the result will be an increased background. To correct for this, a linear background is subtracted. The way to perform the subtraction is not distinct, several possibilities exist. Here it is assumed that the height of the background on the low energy side of a peak should be 1 % of the peak amplitude (from measurements on foils with no mask present). The uncertainty due to the subtraction is estimated to be 10- 15 %.

The beam is normally made homogeneous by using a thin diffuser foil. Due to the energy straggling in this foil, it was not used during the irradiation of the multi-layer sample. Although the beam intensity was made as uniform as possible by defocussing, it may have been inhomogeneous. The relative areas of the peaks in the spectrum may thus be influenced by beam inhomogeneity. The uncertainty due to beam inhomogeneity is estimated to be 10 %. The errors are independent, and can be added quadratically, giving a value of 14-18 %.

Thus, it can be concluded that there is a reasonable agreement between the values achieved by optical measurement and the corresponding using the spectrum evaluation model.

For a real sample the thickness distributions (see fig. 17) will of course be different, the exact positions of the gaussian peaks will not be unique as several fitting solutions are possible. The corresponding thickness distributions will be somewhat different. The uncertainties in the final thick-target corrections will be negligible as, the average thickness of the sample will be correct, and small variations in the shape estimations will have minor effects. This is shown in section 5.2.2 where the effect of erroneous thickness distributions is further discussed.

Which are the limitations and error sources for the method proposed? First, the beam homogeneity is a critical point as discussed above. Second, the collimation must be carefully performed, as stray protons scattered from collimators may cause an undesired background in the spectrum. Third, the carbon resonance is a disturbing phenomenon which to some degree can be corrected for. Using a thin foil of another element with no resonant structure would of course be preferable. Fourth, the energy straggling in the sample and the accompanying peak broadening sets a limit to the minimal sample thickness variation that will be possible to detect. From the spectrum of the multi-layer sample, a value of this can be estimated to be less than 0.25 mg/cm^2 having a thickness of a few mg/cm^2 .

Also, the methods for spectral evaluation is important. The method proposed here for calculating the thickness distribution from the collected spectrum by setting up pure gaussian functions may not give the required accuracy for all types of samples. Low-energy tails may have to be included. In the case of backscattering, a model for the

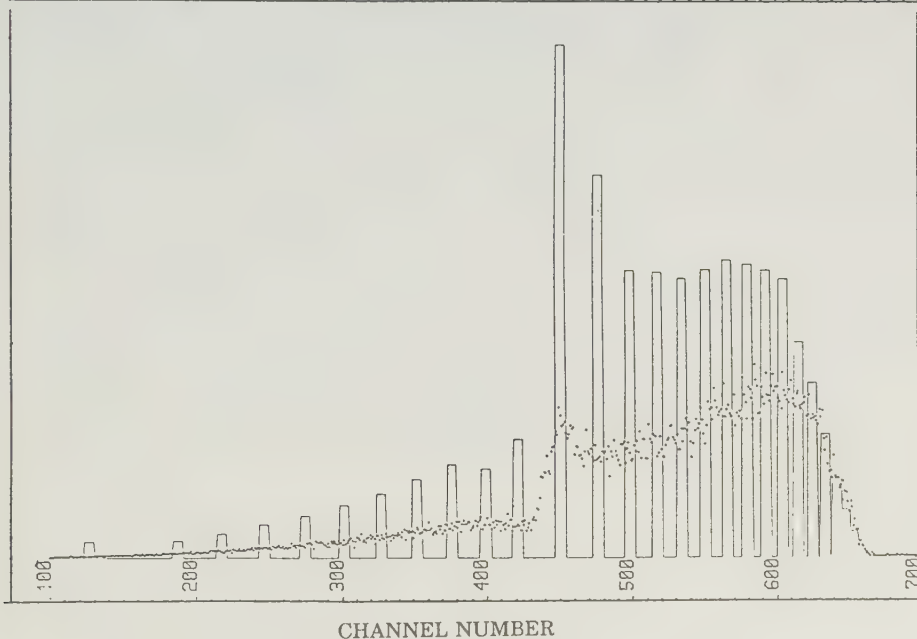
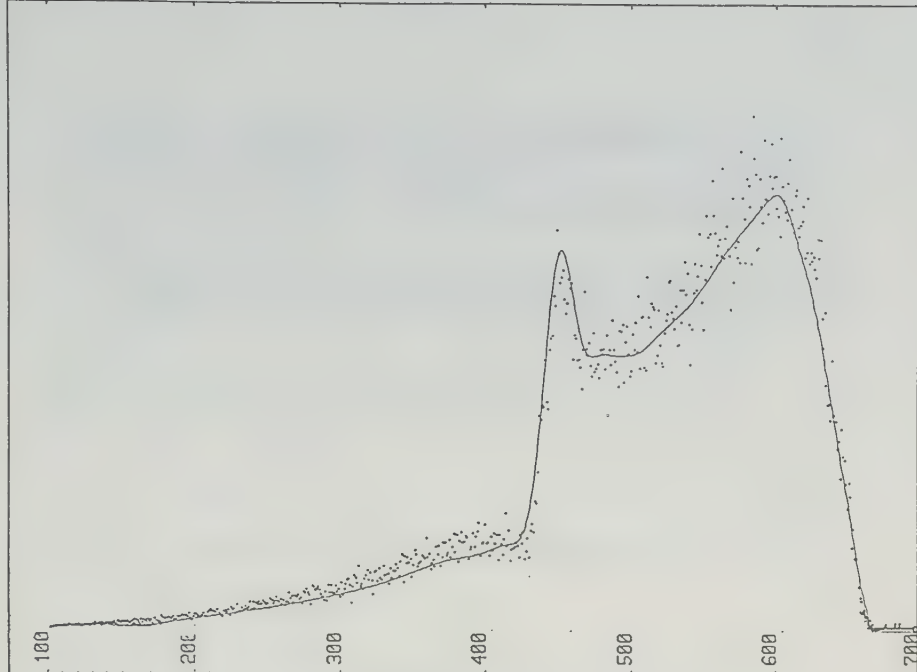


Fig. 17 The spectrum evaluation method applied to a spectrum of a thick inhomogenous biological sample. The points are the spectrum after RESCS correction, the line is the fit of gaussian functions set up. At bottom is shown the histogram representation of the fitted gaussian peak areas, the points are spectrum above.

low-energy tail is given by Mommsen et al.[16,17]. Their model cannot be directly applied to this work. Instead the shape and size of the low-energy tail for a fix geometry can be determined by analysing standard foils of different thicknesses and compositions. The tail can then be parameterized and included into the model.

Finally, it must be pointed out that the main purpose of this method is not to describe the morphology of the sample, but to be a source of relevant target thickness data in order to improve the PIXE quantification procedure.

4. Thick target corrections for a thickness distribution

Having calculated the target thickness distribution, PIXE thick target corrections can be applied in each thickness interval. Assume the following notations:

$k(Z)$ = concentration (g/g) of element number Z in the sample,
 a_i = area of a thickness interval having the areal mass density
 ξ_i [mg/cm^2]
 $c_i(Z)$ thin target PIXE result in a thickness interval, [$\mu\text{g}/\text{cm}^2$]
 $c'_i(Z)$ thick target corrected result [$\mu\text{g}/\text{cm}^2$]
 $f_i(Z) = c'_i(Z)/c_i(Z)$ thick target correction factor for
 this thickness. The thick target corrected result can be written

$$c'_i(Z) = \xi_i k(Z)$$

and $c_i(Z)$, the thin target result can be expressed as

$$c_i(Z) = \frac{c'_i(Z)}{f_i(Z)} = \frac{\xi_i k(Z)}{f_i(Z)}$$

By summing the contributions from all thickness intervals the uncorrected absolute amounts $M(Z)$ [μg] for the irradiated part of the target is obtained

$$M(Z) = \sum_i c_i(Z) a_i = k(Z) \cdot \sum_i \frac{a_i \xi_i}{f_i(Z)}$$

Correspondingly for the thick-target corrected amounts $M'(Z)$

$$M'(Z) = \sum_i c'_i(Z) a_i = k(Z) \cdot \sum_i a_i \xi_i$$

and

$$\frac{M'(Z)}{M(Z)} = \frac{\sum_i a_i \xi_i}{\sum_i \frac{a_i \xi_i}{f_i(Z)}} = F(Z) \quad \text{the thick-target correction factor for the target thickness distribution.}$$

5. Sources of error

5.1 Effects of poor collimation

Scattering of non central protons will broaden the peak. This effect is discussed below. Let r be the distance detector to center of scattering foil and r_x the distance to a point at a distance x from the center on the foil (see fig. 18). Then

$$r_x^2 = (r \cos \alpha)^2 + (r \sin \alpha - x)^2 \quad (5.1)$$

and

$$r_x \cos \alpha_x = r \cos \alpha \quad (5.2)$$

Rewriting (5.1) gives

$$r_x = r \left(1 - \frac{2x \sin \alpha}{r} + \frac{x^2}{r^2} \right)^{0.5} \quad (5.3)$$

Now let x be $\pm d/2$ and combine equations (5.2) and (5.3)

to obtain the extreme scattering angles α_1 and α_2 :

$$\cos \alpha_1 = \frac{\cos \alpha}{\left(1 + \frac{\sin \alpha}{r} d + \frac{d^2}{4 r^2} \right)^{0.5}} \quad (5.4)$$

$$\cos \alpha_2 = \frac{\cos \alpha}{\left(1 - \frac{\sin \alpha}{r} d + \frac{d^2}{4 r^2} \right)^{0.5}} \quad (5.5)$$

Let $L = 50$ mm and $d = 5$ mm. Then $\delta = \arctan(d/2L) \approx 2.86$ degrees of initial scattering in the target. For a scattering angle of 2.86° the kinematic factors for various matrix elements will be:

Z	E_1/E_0
H	0.9974
C	0.9998
N	0.9998
O	0.9998

Next, let $\alpha = 45^\circ$, $d = 5$ mm and $r = 50$ mm. Then $\alpha_1 = 46.9^\circ$ and $\alpha_2 = 42.9^\circ$.

The extreme scattering angles will be $\alpha_1 + \delta = 49.8^\circ$ and $\alpha_2 - \delta = 40.0^\circ$.

The corresponding kinematic factors for various elements are given below.

Angle: 40° 45° 50° (r = 50mm)

H	0.587	0.500	0.413
C	0.961	0.952	0.942
N	0.967	0.959	0.950
O	0.971	0.964	0.956
Cu	0.993	0.991	0.989

which will lead to a very small broadening except for hydrogen. If the detector is closer, e.g. with r = 20 mm the following kinematic factors will be obtained:

Angle: 35° 45° 52° (r = 20 mm)

H	0.671	0.500	0.379
C	0.970	0.952	0.937
N	0.974	0.959	0.946
O	0.977	0.964	0.953
Cu	0.994	0.991	0.988

The Rutherford elastic scattering cross section is a rapidly varying function of angle at the forward angles used here, and this will affect the scattered intensity from the foil reaching the detector. When combining the change in kinematic factor for different scattering angles with the corresponding change in scattering cross section, and taking into consideration that the protons are scattered from a circular surface, the scattering yield versus kinematic factor will show a skew distribution with the maximum at an energy higher than that corresponding to the nominal scattering angle, see fig. 19. Two cases are shown in this figure, corresponding to ratios of the diameter of the irradiated part of the scattering foil to detector distance equal to 0.10 and 0.05. The parts of the curves corresponding to 45 degrees are marked with arrows.

Thus, the maximum of the scattered peak in the spectrum does not exactly correspond to the desired scattering angle. The deviation is a function of the ratio of scattering foil diameter to detector distance. If this ratio is small enough, ≤ 0.05 , the skewness and the broadening effect can be disregarded. Also, as this skewness is only a function of the kinematic factor (if the spectrum is first corrected for the RESCS) and is thus the same in all parts of the spectrum, it will be cancelled during energy calibration.

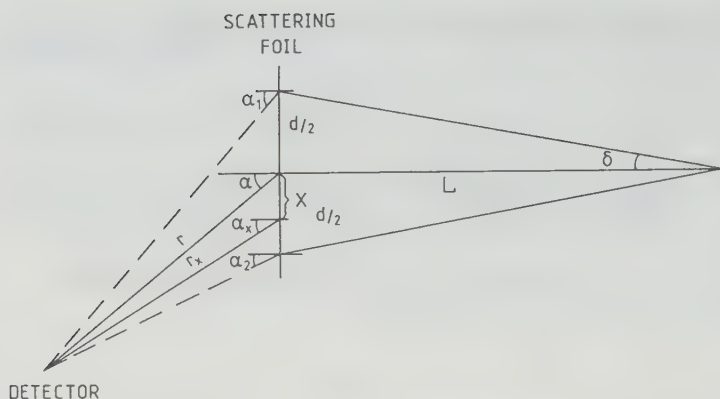


Fig. 18 Definitions of symbols used when calculating the effects of poor collimation. The distance between sample and scattering foil is L , and the distance foil to detector is denoted by r .

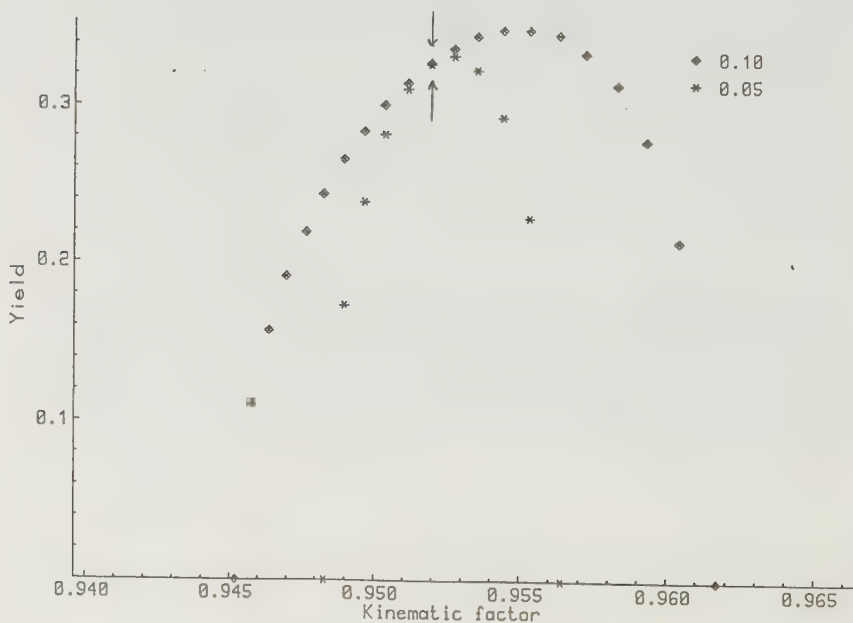


Fig. 19 The scattering yield versus kinematic factor for protons scattered from a circular surface into a detector at 45° . Two cases are shown, the ratio of diameter of scattering foil to distance from foil to detector equals a) 0.10 and b) 0.05. The parts of the curves that correspond to 45° are marked with arrows.

5.2 Thickness correction errors

5.2.1 Erroneous thickness estimation

If the estimation of the sample thickness is poor an error will occur in the PIXE correction factors. An illustration is given in fig. 20 where the deviation is given as a function of target thickness and the deviation from correct thickness for an organic matrix.

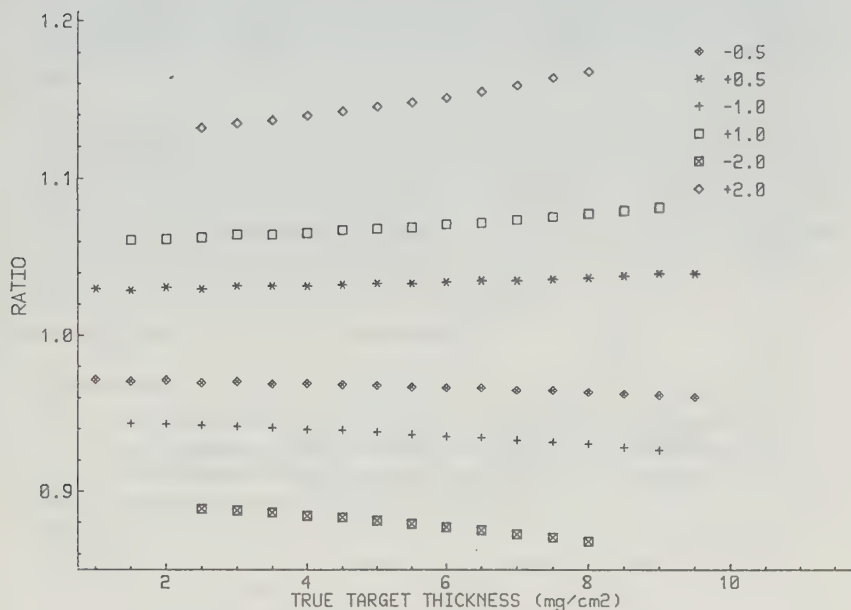


Fig. 20 The ratio of thickness correction factors for a biological matrix with a poor estimation of the real thickness (estimated/true values). The curves show the deviations for thickness discrepancies of ± 0.5 , ± 1.0 and ± 2.0 mg/cm^2 .

5.2.2 Irregular shape

If thick-target corrections are made to a target with an irregular thickness distribution, assuming a constant thickness, errors will arise. Here some illustrations are given, see fig. 21. Case a) Assuming a flat thickness distribution when in fact the thickness increases step-wise. The resulting errors have been calculated for $\xi = 10$ mg/cm^2 . Case b) and c) Assuming a flat thickness distribution when the sample thickness varies as shown in fig. 22, the "tower" distribution. In cases a) and b) the mean thickness of the target is correctly assumed, while in case c) it is estimated to be 2 mg/cm^2 instead of 4 mg/cm^2 .

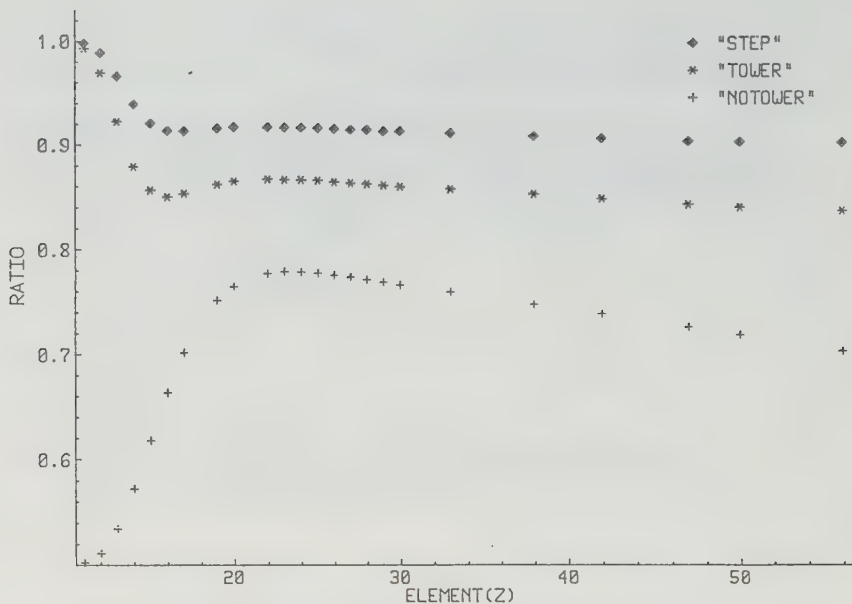


Fig. 21 The ratio in thick-target correction factors when assuming "flat" target distributions (6 mg/cm^2 , 4 mg/cm^2 and 2 mg/cm^2) instead of the real "STEP" and "TOWER" distributions for a biological matrix. The points marked "NOTOWER" show the case where the mean thickness also is underestimated (2 mg/cm^2 instead of 4 mg/cm^2).

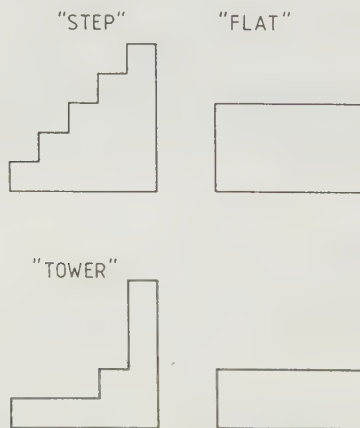


Fig. 22 Example of a targets with various thickness distributions, "step", with a step-wise (2 mg/cm^2) increasing thickness, "tower", one thick part (10 mg/cm^2) and the rest thinner (2 mg/cm^2), and "flat", constant thickness.

5.3 Discussion

It can be seen that the deviations for these cases can be 10 - 20 % even if the average thickness is correct. There is a smoothing effect in the thickness correction factors. However, if the mean thickness is incorrectly estimated, as would be the case where one part of the sample was much thicker than the rest and this is not known, there will be larger errors. A practical example of this is the case of solutions pipetted on a foil. After drying, small but thick structures of crystals or rings are formed. Knowing the mean value of the target thickness is a point of major importance. To determine this one must know the centre of mass of the target thickness distribution, which requires the performance of all the steps described in section 3.3.2.

When trying to improve the accuracy and precision in the PIXE-analysis it is important to take the thickness variation into account and perform the thickness corrections as described in section 4. The resulting thickness distributions can contain 30-50 thickness intervals. For practical use, this division is too fine, and it is possible to average the distribution over larger intervals. The criterion for the averaging is based upon the degree of accuracy and precision required in the final PIXE-results, taking into account the degree of skewness or variability in the thickness distribution.

6. Summary

It is essential to know and if necessary to correct for the target thickness in order to be able to perform good PIXE analysis. If the sample has an irregular thickness distribution it is also important to know mean thickness, which can be determined using the method described in this work. It uses the particle spectrum obtained when particles (in this work protons), having passed through the target, are forward scattered in a thin carbon foil into a detector at 45° .

It is proposed that the spectrum contains information about the target thickness distribution. By correcting the spectrum for the Rutherford elastic scattering cross section, and building up the corrected spectrum as a sum of gaussian peaks having a FWHM varying in accordance to the energy straggling, an energy histogram is obtained. By relating the energy loss in the target to the corresponding target composition, the energy distribution is transformed into a target thickness distribution.

The spectrum evaluation model is demonstrated on a sample with a stepwise increasing thickness, and reasonable agreement is found. Some further improvements to be made include a more detailed study of the background and the low energy tails.

Using calculated target thickness distribution renders a more detailed thick target correction possible, thus increasing the general accuracy and precision of the PIXE analysis.

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Appendix

The code THICK-500

The code uses the polynomial fit given by Akselsson and Johansson [18] for X-ray ionization cross sections, stopping power data from Northcliffe and Schilling [19] and X-ray attenuation data from Veigele [20]. The thick target factors are calculated by slicing the target into layers of equal energy-loss. The numbers of slices varies between 100 and 200. For each layer is computed its areal mass density, the proton energy in the the centre of the layer, the corresponding X-ray production cross section and for the X-rays created in the layer their absorption during the path to the target surface. The contributions from all layers are than added together.

For completely thick targets the correction factors will convert the thin-target results [ng/cm^2] into concentrations [ppm], for targets of intermediate thickness into areal concentration [ng/cm^2].

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